PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

IV. The Negative Acceleration of Growth with Age as Demonstrated by Tissue Cultures.

By ALFRED E. COHN, M.D., and HENRY A. MURRAY, Jr., M.D.
With the Assistance of Alma Rosenthal.
(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, February 26, 1925.)

It has been shown by weighing chicken embryos that the percentage rate of growth decreased with age (1). The experiments to be now reported were planned so as to determine whether the growth rate and the latent period of growth of fragments of tissue removed from the hearts of embryos varied with age in the same fashion. In other words, are functional changes that take place in the organism as a whole mirrored by similar changes in individual cells? Do the cells themselves undergo differentiations in form and function so that when removed from the organism they exhibit characteristics of life which vary in kind or quantity according to the age of the embryo from which they were derived? The rate of division is one character by which cells of different ages may be compared.

By implanting pieces of tissue of approximately equal mass from corresponding sections of the heart into similar culture media an answer to this question may be obtained. Here the independent variable is the incubation age of the chick. That the enlargement of the area which occurs under certain conditions of tissue culture is not simply the result of cell migration has been shown by Burrows (2) and Carrel (3). Accordingly we were led to believe that growth phenomena could be investigated by this method.

It soon became evident that values for growth rate comparable to those found in studies of the embryo as a whole could not be obtained by the tissue culture method without making certain arbitrary and

unjustifiable assumptions. It was found that the differences between the growth indices of tissues of different ages varied inversely with the length of the period of cultivation. For instance a fragment from a 4 day embryo after 24 hours had grown 15 times as much as a similar piece from a 16 day embryo; whereas after 4 days the growth area in the younger piece was only about 5 times that from the older section. Although some arbitrary time could have been chosen for measurement which would give values for growth rate change similar to the figures already described as having been obtained for the whole embryo, there were no facts to justify us in this or any other selection of a time interval. It was evident that for a positive result we could hope for a tissue culture growth curve which in its main characteristics only, duplicated the curve for the whole embryo.

Carrel has shown that the rate of growth of a standard 11 year old strain of fibroblasts was affected by the age of the hen from which the plasma culture medium was derived (4). The older the donor the lower was the growth index; that is to say, the slower the growth of the transplanted tissue.

The present investigation concerns itself with the other aspect of the problem; namely, the rate of growth of tissue from embryos of different ages in a standard plasma medium. There are numerous factors of error, some inherent in the material and others related to our tissue culture technique. For instance we have chosen arbitrarily the ventricular wall of the heart as the site for dissection and have cut pieces about 0.4 sq. cm. in size. As the embryo develops, however, the cardiac wall becomes more compact, the muscular nature of its structure becomes more marked, and the relative proportion of endo-, meso-, and pericardium is altered. The thickness of the wall also varies. For these reasons it is impossible to cut fragments of identical composition or size in embryos of different ages. In view of the fact, moreover, that the different organs of the body are developing at varying rates, the growth rate of cardiac muscle may be no criterion of general cellular behavior or the rate of aging in the whole embryo. This seems to be a theoretical objection, but may be irrelevant since the growth from these fragments consists mostly of fibroblasts, which are relatively undifferentiated and probably of a like nature, metabolic rate, etc., throughout the body. It was thought that the medium

chosen as a standard might have a differential effect on tissues of varying ages; in other words as this study is concerned with the change in rate of growth with age, there is an error inherent in the use of any medium other than the natural one. If the natural *milieu* were used, however, one could not differentiate cellular from environmental effects. One advantage of choosing the tissue rather than the plasma as a measure of age is that tissues can be easily obtained from a chick at an early period of embryonic life whereas it is not possible to collect plasma so early. The studies comprising this series are limited to the embryonal period. Since it is well known that the period of greatest negative growth acceleration occurs at the inception of life (5) this most important part of the cycle could not be included if the investigation were limited to the influences of plasma. Finally it is obvious that as soon as the tissue has been transplanted and has started to develop it is no longer identical with the tissue *in vivo*. It becomes dedifferentiated one might say. The significance of the experiment is necessarily limited by these conditions.

Method.

Our method was so similar to Dr. Carrel's that it would seem sufficient to give the publications of Carrel and Ebeling as reference (6). Dr. Carrel put at our disposal all his knowledge dealing with the technique of tissue culture. We should like to take this opportunity to express our appreciation of his assistance and goodwill.

As a review, however, and because in some ways the procedure was slightly modified, it would not be inappropriate to outline our technique for growing tissue fragments from the ventricular wall of embryo chicks of different ages. The method of collecting, handling, and incubating the eggs has been described elsewhere (7).

Only fresh blood is used. On the morning of the experiment a hen approximately 1 year old and bred from the same stock which provides the eggs is bled from the carotid artery through a paraffined cannula into iced paraffined tubes. These are centrifuged and the plasma which furnishes the culture medium for that day is removed by a pipette. The experiment is carried on under sterile precautions, the linen, gowns, etc., being sterilized by steam and the instruments and glassware by dry heat (at 145–155° for 1 hour). The glassware such as dishes, watch-glasses, pipettes, and test-tubes is all of Pyrex or other glass relatively free from alkali. This does not apply to the so called Gabritschewski dishes which do not come in contact with the cultures or medium at any time. The tissues are planted on mica cover-glasses.

The experiment commences with the preparation of extract. Two 10 day old embryos are cut up into small bits, 1 cc. of Ringer's solution is added, the whole is centrifuged for 10 minutes, and the supernatant fluid (avoiding a layer of tissue debris which is usually found floating on top) is pipetted off. Whenever tissue extract is mentioned it refers to this preparation.

The ages of the embryos range from 4 to 18 days of incubation. The hearts are removed and dissected in Ringer's solution on Pyrex glass slabs with an iridectomy scalpel. Under the glass dish is a piece of black coordinate paper which guides one in cutting sections of the proper size. Only those plates were included the fragments of which averaged between 0.310 and 0.490 sq. mm. The variations in size between these limits are rendered insignificant by the method of estimating growth. Ten small fragments are transferred from the dissecting dish to the mica cover-glass in a pipette, the excess of Ringer's solution removed, and 0.5 cc. of chicken plasma added. It is spread out so as to cover a circle 4.4 cm. in diameter etched on the mica. This provides for an equal number of pieces (ten) in an equal quantity of the medium (0.5 cc.) distributed over an equal surface (15.2 sq. cm.) and therefore of approximately equal thickness. The tip of the knife dipped as far as a certain point into the tissue extract preparation is used to stir the medium and distribute the fragments equally throughout the plasma. The minimal amount of tissue extract thus introduced has some effect in accelerating growth, but it is used principally to insure the formation of a good clot.

When clotting has occurred the mica is inverted over the Gabritschewski dish and sealed with paraffin and vaseline. All the fragments in one dish are of one age. In the case of the 4 and of the 5 day embryos two hearts are necessary to provide ten pieces. In the older chicks, on the other hand, two plates were occasionally planted with fragments from the same heart.

The fragment and the peripheral growth were measured every 24 hours until enlargement ceased by drawing the projected image of it, magnified 27 times, and measuring the area circumscribed in the drawing with a planimeter. As the depth of growth could not be accurately determined, development was estimated by comparing areas.

We do not know whether growth (enlargement of area) is a function of the area or of the circumference. Theoretical considerations might suggest the latter, since there is no limiting surface and the internal growth, that is to say the swelling, or cell division within the original fragment has little influence on the marginal radiations. Empirically we tried to settle the point in the following manner. Twelve plates were planted with similar technique. There were 110 pieces of tissue in all (six to ten fragments planted in each dish). The area of the fragments in each dish varied from 0.288 to 0.452 sq. mm. in size, the average size being 0.382 sq. mm. The incubation ages of the embryos from which the tissue was removed varied from 4 to 14 days.

Growth was measured in the usual fashion, and the index of growth was estimated by four different methods for each of the 110 fragments after 24 hours and then again after 48 hours (Fig. 1). The methods employed for finding a value for growth rate were: (1) subtracting from the total area of tissue after n days of cultivation (A_n) the area of the central portion after n days of cultivation (B_n) (which was not usually of the same size as the original fragment since the latter may

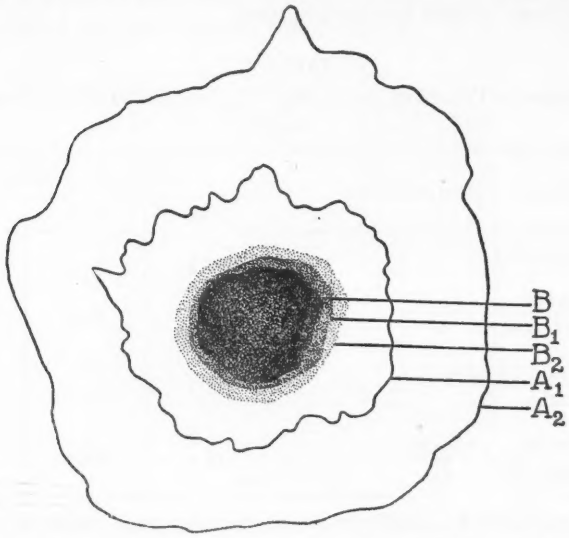


FIG. 1. A schema to show the enlargement of the implanted fragment (B) after 1 day (B_1) and after 2 days (B_2); and the margins of the new growth after 1 day (A_1) and after 2 days (A_2). (*Cf.* Table I.)

enlarge or in some cases contract), (2) dividing the growth area as just described ($A_n - B_n$) by the size of the original fragment (B), (3) dividing the growth area ($A_n - B_n$) by the area of the central fragment at the time of measurement (B_n), (4) dividing the growth area ($A_n - B_n$) by the square root of the central fragment (B_n).

The coefficient of variability[1] for each of these methods is given in

[1] The coefficient of variability is the standard deviation relative to the mean value; *i.e.*, $Cv = \frac{100\sigma}{M}$.

Table I. It can be seen that no method shows any conspicuous advantage over any other. We finally used as an index of growth the formula:

$$\frac{A_n - B_1}{B_1} \text{ or } \frac{A_n}{B_1} - 1$$

We determined the coefficient of variability at infrequent intervals in subsequent experiments, and found that as our technique improved the variations became less pronounced.

TABLE I.

The Coefficient of Variability for Various Methods of Measuring Tissue Culture Growths.

Method for obtaining growth index.	Coefficient of variability.*		
	24 hrs.	48 hrs.	Average.
1. Growth (A_n-B_n)	41.8	30.4	36.1
2. $\frac{\text{Growth}}{\text{Original fragment}}$ $\frac{(A_n-B_n)}{B}$	44.3	30.8	37.5
3. $\frac{\text{Growth}}{\text{Central area}}$ $\frac{(A_n-B_n)}{B_n}$	43.1	31.2	37.1
4. $\frac{\text{Growth}}{\sqrt{\text{Central area}}}$ $\frac{(A_n-B_n)}{\sqrt{B_n}}$	42.0	28.1	35.0

* The coefficient of variability is the standard deviation relative to the mean value; *i.e.*, $Cv = \frac{100\sigma}{M}$.

RESULTS.

The accompanying curve (Fig. 2) shows that the growth rate and the decline in the growth rate (*i.e.*, negative acceleration) after 48 hours of cultivation are greatest for the early days of life and, except during a short period when there is an apparent increase, decrease with age. We considered that our culture medium, namely plasma from a 1 year old hen, might exercise a differential effect on pieces of different ages since it was arbitrarily selected and did not represent the normal environment of the tissue. For instance it might be more suitable for the older than for the younger pieces. We performed a

few experiments with plasma from hens 2 and 3 years old and obtained comparable figures. This change in the age of the plasma apparently had no marked differential effect on growth rate. As plasma from young embryos cannot be obtained with facility the reproduction of a more natural environment was not attempted. We did perform a series of experiments in which the plasma was diluted with equal parts of Ringer's solution (Table II). This change seemed to increase markedly the rate of growth of the older fragments, but the curve was nevertheless of the same general shape (Fig. 3).

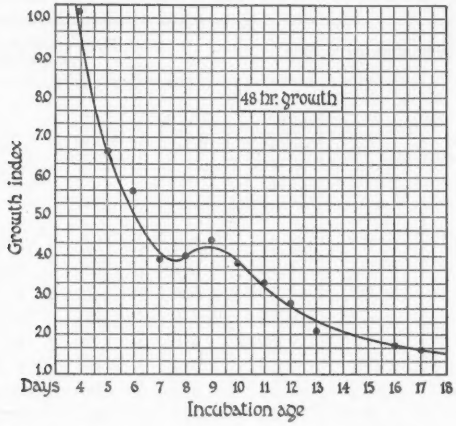


FIG. 2. The tissue culture growth rate, as approximately represented by the growth index after 48 hours—

$$\frac{\text{Total area (2 days)} - \text{area of original fragment after 1 day}}{\text{Area of original fragment after 1 day}} = \frac{A_2 - B_1}{B_1}$$

Plotted as a function of embryonic age. Abscissæ are the ages of the embryo from which the fragments were derived (Table II).

In all these graphs there appears to be a deflection, or "hump," between the 7th and 11th days in an otherwise smooth curve. Speculation as to the nature and cause of this feature will be postponed until certain changes occurring at this age in the physiology of the heart have been investigated.

In Table III have been collected the average results for each plate with their deviations. If these are equated against age (Fig. 4) it

will be found that no better smooth curve can be drawn to describe them than the one derived from the formula $\frac{\frac{dw}{dt}}{W} = 3.6\,t$. The general shape of the tissue culture curve is accordingly seen to be similar to that found to be representative of growth changes in the embryo as a whole.

TABLE II.

The Growth of Tissue Cultures in Undiluted and Diluted Plasma as a Function of the Age of the Embryo from Which Fragments Were Removed.

Undiluted plasma medium.					Plasma medium diluted with equal parts Ringer's solution.		
1 Age of embryo.	2 No. of fragments.	3 B_1 Average area of original section after 1 day.	4 1 day. Growth index, $\frac{A_1 - B_1}{B_1}$.	5 2 days. Growth index, $\frac{A_2 - B_1}{B_1}$.	6 No. of fragments.	7 B_1 Average area of original sections after 1 day.	8 Growth index, $\frac{A_1 - B_1}{B_1}$.
days		*sq. mm.*				*sq. mm.*	
4	95	0.377	3.11	10.2	12	0.316	2.74
5	100	0.411	1.34	6.6	10	0.362	1.40
6	115	0.342	1.19	5.6	7	0.322	1.53
7	111	0.372	0.90	3.9	35	0.417	1.46
8	67	0.487	0.80	4.0	8	0.410	1.71
9	118	0.369	0.95	4.4	47	0.367	1.49
10	96	0.372	0.76	3.8	26	0.388	1.19
11	57	0.362	0.51	3.3	25	0.367	1.22
12	98	0.391	0.45	2.8			
13	88	0.361	0.30	2.1	20	0.351	1.11
14	38	0.352	0.27	2.9	20	0.378	1.27
16	40	0.321	0.16	1.7	19	0.351	0.73
17	35	0.330	0.03	1.6			
18					7	0.315	0.61

Curves to show the change in percentage growth during the period of cultivation are shown in Fig. 5. The growth is expressed as the difference between the logarithm of the whole area and the logarithm of the original fragment $\left(\log\left[\frac{A_n}{B}\right]\right)$. All pieces may be started on the base line. Each point represents the average value of ten sections (Table IV). The slope of the line is equivalent to the percentage rate of growth. The curves are representative of others. It seems

that the maximum growth rate of the younger pieces is reached at the very commencement of their history, whereas the oldest fragments do not attain their greatest velocity until later; that is to say, after about 1½ days. All the pieces stopped growing about the same time.

Latent Period.

The time intervening between the incubation of the cultures and the first appearance of cells protruding from the peripheral margin is

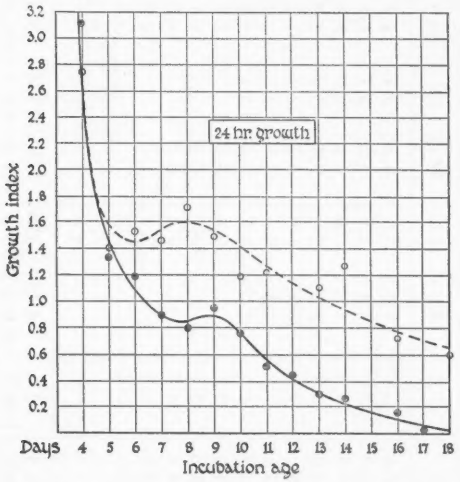


FIG. 3. The growth index after 24 hours in chicken plasma (⊙——⊙), and in plasma diluted with Ringer's solution (○---○) equated against embryonic age (Table II).

termed the latent period. A satisfactory interpretation of the processes involved during the latent period has never been rendered, either for tissue cultures or for analogous experiments, such as the transplantation of bacteria and yeast colonies into a new environment. In studying the growth of bacteria in fresh media it has been found that, unless special precautions are taken (8), soon after planting the number of viable organisms in the culture becomes diminished (9). It is only somewhat later that the growth and multiplication of the colonies commence. The latent period has been taken as the time

TABLE III.

Average Growth Index Figures with Standard Deviations for Each Age.

Days.....	4	5	6	7	8	9	10	11	12	13	14	16	17
Average growth index for each culture plate* after 48 hrs.	10.0	6.2	7.4	3.8	5.6	5.7	3.5	3.7	3.4	3.8	2.5	1.3	1.2
	9.0	4.2	5.3	5.8	4.4	1.9	5.2	2.7	3.7	2.3	2.6	1.0	2.2
	12.0	6.7	5.5	4.5	3.8	7.0	4.8	3.9	3.8	2.0	3.8	1.7	2.2
	8.6	4.9	3.9	3.9	3.3	3.4	5.4	2.9	3.3	1.9	2.8	1.5	0.7
	8.1	3.2	6.0	4.5	3.8	1.9	4.3	2.8	3.5	3.4		4.0	
	8.0	5.4	5.1	2.1	4.5	5.3	3.7	3.6	2.1	1.0			
	11.2	12.6	4.5	1.9	3.3	4.7	5.6	3.4	3.7	2.0			
	13.7	7.7	4.3	3.9		5.4	3.3		2.1	1.0			
	14.2	8.5	5.6	3.7		4.7	2.5		2.1	3.4			
	7.4	4.7	4.8	4.7		5.7	2.0		4.3				
	8.4	6.3	4.8	2.5		7.1			2.0				
			8.1	3.2		4.4			2.4				
						1.9							
						2.9							
Average†.....	10.0 ±1.5	6.4 ±0.8	5.4 ±0.8	3.7 ±0.7	4.1 ±0.5	4.9 ±1.2	4.0 ±0.8	3.3 ±0.3	3.0 ±0.5	2.3 ±0.7	2.9 ±0.2	1.9 ±0.7	1.6 ±0.4

* Usually ten fragments in each plate.

† The averages are given ± the standard deviation.

intervening between the plantation of the culture and that moment at which the original number of living bacteria is again estimated to be present. In the case of tissue cultures we had no quantitative method for estimating cellular death after transplantation and therefore this phase of negative growth which has been clearly demonstrated for bacteria is not apparent. At present we shall simply show how

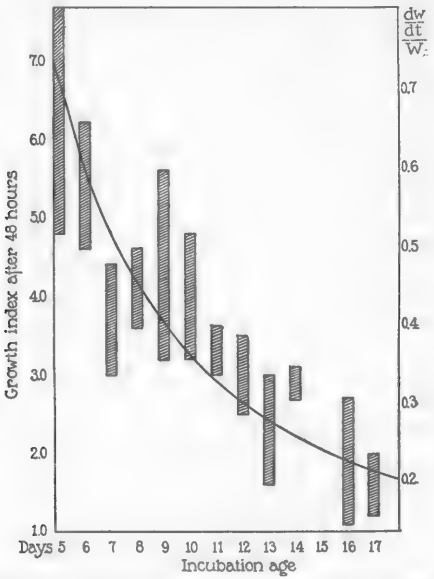


FIG. 4. The growth index ± the standard deviation (Table III) plotted against the age of the embryo. The smooth curve corresponds to the ordinates on the right hand side, which are for $\frac{dw}{dt}/W$; *i.e.*, the percentage growth rate of the embryo as a whole over the same period (*cf.* Murray (1)).

the latent period is related to the age of the tissue. It should be compared with the curve showing the growth index as a function of age. The fact that these two curves are dissimilar would seem to indicate that the phenomena in question are of a different nature. The latent period as measured in these experiments corresponds roughly to the term reactivity or irritability, the stimulus being the

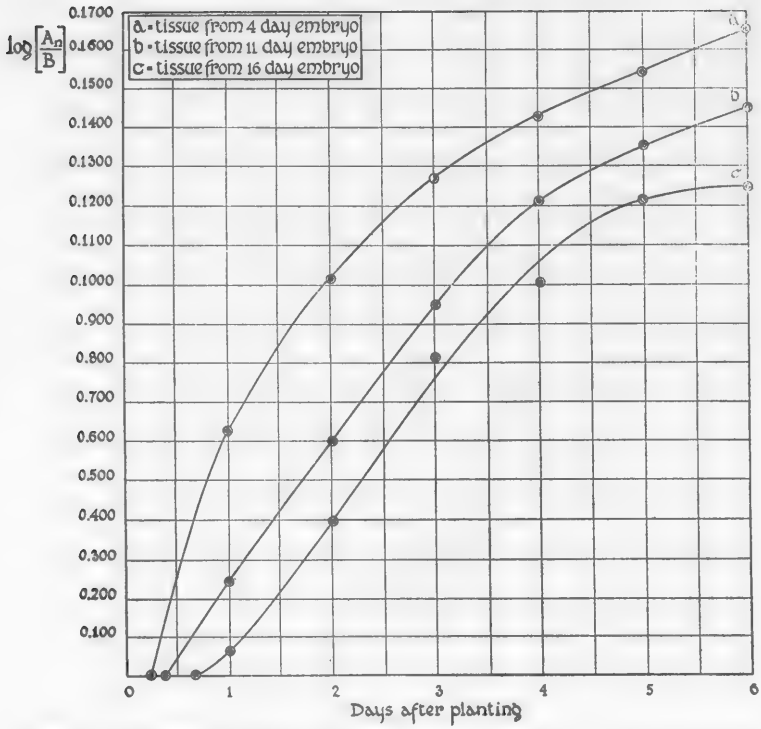


FIG. 5. The growth in culture of tissues from embryos of 4, 11, and 16 days of age respectively over a 6 day period. The ordinates are the logarithms of the percentage increase in area; *i.e.*, $\log\left[\frac{A_n}{B}\right]$. The abscissæ represent the duration of cultivation in days (Table IV).

TABLE IV.

Percentage Growth of Tissue Cultures for the 6 Day Period.

No. of plate.	Age of embryo.	Log percentage growth (*i.e.* $\log\left[\frac{A_n}{B_1}\right]$; *i.e.*, $\log A_n - \log B_1$). Days after planting.					
		1	2	3	4	5	6
	days						
R 113	4	0.622	1.004	1.262	1.418	1.531	1.646
R 120	11	0.238	0.592	0.943	1.205	1.348	1.438
M 139	16	0.062	0.386	0.804	0.996	1.205	1.237

incision and the response being the initial growth of cells from the periphery. In another paper it will be seen that the latent period seems to be correlated to the chemical form or constitution of the tissues rather than to the rate of growth.

The technique employed was the same as that used in the growth experiments.

Within about 5 minutes of planting the plate was placed, mica side up, in a Freas electric oven where the temperature was kept at about 38°C., a rather narrow temperature fluctuation being maintained by the use of an electric fan in the incubator. The plates were then regularly observed at varying intervals under

TABLE V.

Latent Period of Tissue Cultures.

Undiluted plasma.			Plasma diluted with Ringer's solution.		
Age of embryo.	No. of fragments.	Average time.	Age of embryo.	No. of fragments.	Average time.
days		*hrs.*	*days*		*hrs.*
4	52	5.5	4	20	4.9
5	90	5.7	5	30	5.3
6	111	5.8	6	29	4.9
7	88	5.9	7	40	5.6
8	56	6.0	8	50	5.5
9	83	6.7	11	30	6.9
10	60	7.7	13	10	7.8
11	50	9.3	14	20	9.3
12	71	10.4	18	10	11.0
13	69	11.7			
16	42	16.1			

low and high power magnification in an observation incubator. The arbitrary point chosen as terminating the latent period was the moment when at least three young cells could be clearly identified sprouting out at different points on the periphery. The average time interval in hours (for the ten fragments in each plate) before this amount of growth was noted was taken as the latent period for an embryo of the age involved (Table V). Several plates were prepared for each age.

The averages for the latent period are shown graphically in Fig. 6. The effect of Ringer's solution as a plasma diluent in diminishing the latent period of the older pieces is clearly shown. This action is not enough, however, to account for the increase in the growth rate

shown to occur in previous experiments when Ringer's solution was added to the culture medium.

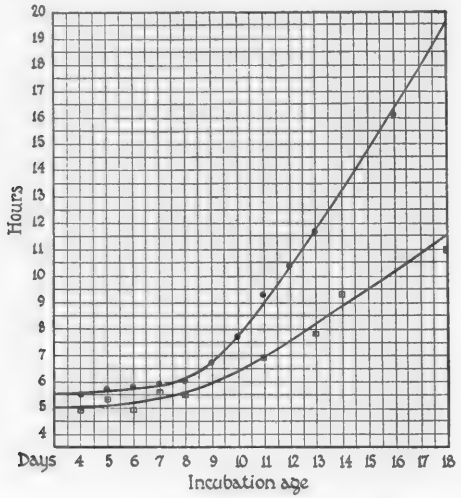


FIG. 6. The latent period of growth of fragments of heart muscle from chicken embryos of different ages when implanted in plasma (⊙) and in plasma diluted with Ringer's solution (⊡) (Table V). The ordinates represent the latent period in hours and the abscissæ the age in days.

DISCUSSION.

The results summarized in this paper, based upon the statistical average of over 1,000 heart muscle fragments of different ages planted and incubated under similar conditions, indicate that there is an increase in the latent period with age, most marked in the latter half of the embryonic cycle, and a decrease in the growth index, most marked in the beginning of life. In its general configuration the curve for the growth rate of tissue cultures resembles the curve for the organism as a whole except that there is a rise between the 7th and 11th days, for which we have at present no explanation. It may be due to the greater susceptibility to a foreign medium of the younger pieces or the result of factors specific to the heart.

From these observations the inference may be drawn that in a

multicellular organism the divisional velocity of the individual cells decreases progressively with age. It is not yet apparent how the separate components, that is to say the divisional rate (increase in number) and simple growth rate (increase in mass), are related to one another. The organization of the body as a whole becomes more defined with time, imposing upon cells certain habits which are in a sense acquired characters. Of these habits the one that concerns us at present is that which involves the rate of division and increase in mass. The habit that is acquired and inherited is one of constantly decreasing growth rate. When older tissue, as for instance a heart fragment from a 16 day old chicken embryo, is placed in a fresh environment, it does not assume immediately a divisional velocity typical of younger tissue. Under the conditions of our experiment the previous growth rate was not approached for 36 hours. In other words a certain number of cell divisions (*i.e.* generations) were required for it to lose the habit of slow growth previously imposed upon it by its organized environment. The happening is an expression of a process of rejuvenescence or dedifferentiation. The tissue soon assumes a growth rate determined by its environment, and as long as the environment can be kept relatively stable, that is to say of uniform composition, presumably there will be no further change in divisional velocity.

It is clear that the organism as a whole and probably each and every part, fixed and wandering cells and circulating fluids, all show variations with age which act to condition growth rate. The changes in the plasma, however, are obviously the results of changes in cellular activity throughout the body. For, do not nutritive substances, whence the body derives its energy, depend upon body cells for their absorption and preparation? And is not the concentration in the blood of various chemical substances, such as hormones, a function of the activity of various gland cells? And does not the elimination of injurious substances depend upon the viability of specific excretory tissues? Thus, it is that the *milieu intérieur* is determined by cellular dynamics and *vice versa*. The relationship is reciprocal.

Whatever be the chemical nature of the various products derived from body tissues and fluids which modify the growth activity of cells under experimental conditions their concentration in the body is

certainly a function of the metabolic activity of the living protoplasm. If these substances become decreased or increased with age or if new substances develop in the course of time, the conditions for the variation must be sought for in the tissues themselves.

SUMMARY.

1. Observations on tissue cultures of heart muscle from chicken embryos indicate that in general the growth rate of the individual cells decreases with age. The negative acceleration of growth is greatest at the beginning of life as in the intact embryo.

2. The latent period before growth commences increases with the age of the implanted tissue, but in a different fashion. The acceleration of the latent period is greatest near the *end* of the incubation period, thus demonstrating that the factors determining the *initiation* of growth and those determining the *extent* of growth are not similar.

BIBLIOGRAPHY.

1. Murray, H. A., Jr., *J. Gen. Physiol.*, 1925–26, ix, 39.
2. Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.
3. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxvii, 759.
4. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.
5. Minot, C. S., The problem of age, growth and death, New York, 1908.
6. Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.
7. Murray, H. A., Jr., *J. Gen. Physiol.*, 1925–26, ix, 1.
8. Morgan, H. J., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 207.
9. Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

V. On the Rate of the Heart Beat during the Development of Chicken Embryos.

By ALFRED E. COHN, M.D.

With the Assistance of Edith L. Wile.*

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 2, 1925.)

A function so intimately associated with the developing life of the organism as is the rate of the heart beat should be studied exactly. It may have importance from the point of view of the development of the whole organism and its metabolism, of the circulation, and of the differentiation of the function of stimulus production on the part of the various structures which ultimately form the adult heart.

A systematic study of the pulse rate throughout the embryonic cycle of the life of the chicken does not seem before to have been undertaken. Wernicke (1) made two series of partial observations for this purpose, one between the 46th hour and 7th day in which the attempt was made to maintain the temperature constant by means of a sand bath, and a second between 67 hours and 11 days in which the shell and outer shell membrane were removed and the whole egg, embryo and yolk, placed in a water bath. In both series large numbers of experiments were performed at each age investigated, but when the data are plotted a consistent rate curve cannot be drawn through the points, even when cases of like temperature are compared. Why order cannot be made from these experiments it is difficult, perhaps impossible, to understand.

In the observations reported it was our purpose to carry on the study in such a manner that, if there were an underlying order in the rates which the heart develops at succeeding stages throughout its embryonic life, that fact would emerge from the information collected.

* We are indebted to Miss Alma Rosenthal for aid in the early part of these experiments.

It seemed to be important to take care not only of the conditions within the incubator in which the eggs developed but more important still of the room in which the observations were made. After futile attempts had been made to work in an ordinary room at ordinary room temperature, a room already described (2) was built in which a constant temperature (38°C. ± 1.0°) could be maintained. The eggs were of white leghorn hens, obtained from the source already indicated. They were incubated under standard conditions (2). In order to count the rate of the heart a small window was cut or picked out of the shell, care having been taken, by maintaining the position constant during the preceding 12 hours and by candling, to ascertain the position of the embryo. Counting was facilitated by observing the heart itself in the younger embryos through a binocular dissecting microscope. The field was illuminated by focusing a beam of light on the window and the tissues within. In order to eliminate the influence of heat rays a water bath through which a stream of cold running water was directed was interposed in the course of the light beam. In the older embryos when the heart was no longer visible the count was obtained either by observing the pulsation of a vessel, or of the stream within the vessel, or sometimes the motions of a beam of light to which the pulsations of the heart or of a vessel were communicated. Counts of half a minute's duration each were made for about 5 minutes.

To estimate the stage of development at which the embryo had arrived we used four means. (1) We knew the incubation time; (2) we weighed the embryos after the counts were made; (3) we made drawings of the area of the heart and measured these; and (4) we compared the morphology of the heart with a series of drawings of hearts of known age at successive periods. The drawings for this last estimation were made in our laboratory during the course of this and of other work and they were checked against the data found in the literature. As will be seen later, the variation in the heart rate at any given stage was so great that the use of it in an attempt to be precise in respect to the state of development was abandoned. In the end weighing proved the best control. If the average weight at each age of the embryos in this series is compared with Murray's (3) curve showing the relation of weight to age a close correspondence between our findings and his is evident (Fig. 1).

The age can apparently be established also by measuring the area of the heart. The hearts were excised, after the embryos had been weighed, and were floated, to avoid anteroposterior flattening, in chicken serum contained in a receptacle formed by a glass ring secured to a glass slide by paraffin. The outline of the

heart was drawn by means of an Abbe drawing apparatus. Directly afterward, a stage micrometer was placed on the microscope stage at the level where the heart had been and its scale was drawn on the same paper as the outline of the heart. The magnification of the area of the heart was later computed from the drawing. If the logarithms of the areas (× 10) so found are plotted against the

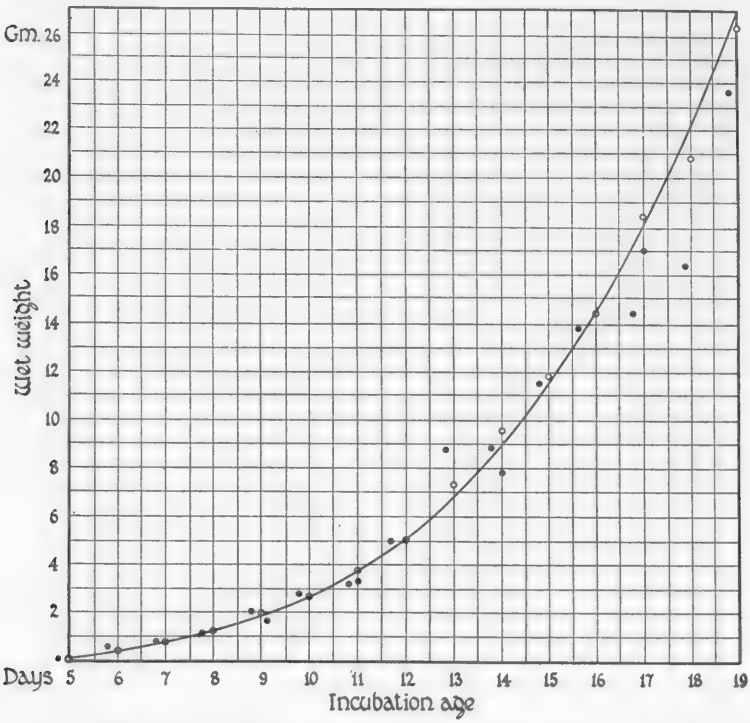


FIG. 1. The curve and the white circles (○) shown are the same as those of Fig. 1 in Paper III of this series (3). The solid black circles (●) are the average weights at the ages indicated of the embryos studied in this series.

age a smooth curve (Fig. 2) can be drawn through points. It is apparent that the greatest rate of increase in the area of the heart, as in the weight of the embryo, is found in the first 4 days. It is clear, then, that the age of embryos can be known in terms either of the weight of the embryo, as Murray has shown, or of the area of the heart, and that these measurements are apparently functions of the incubation time.

No more than a qualitative statement can be made on the relation of the

morphology of the heart to age. Before the 7th day the estimate that was made of the probable age of the embryos based on comparison with the drawings corresponded closely with the age as ascertained by the other means that were employed. After this time, the changes in form are not sufficiently sharp within brief periods of time to permit judgments of even approximate value to be made.

It proved possible in the manner just described to establish a parallel between the weight, cardiac area, and incubation age of the embryos and throughout the work this was carefully done. The

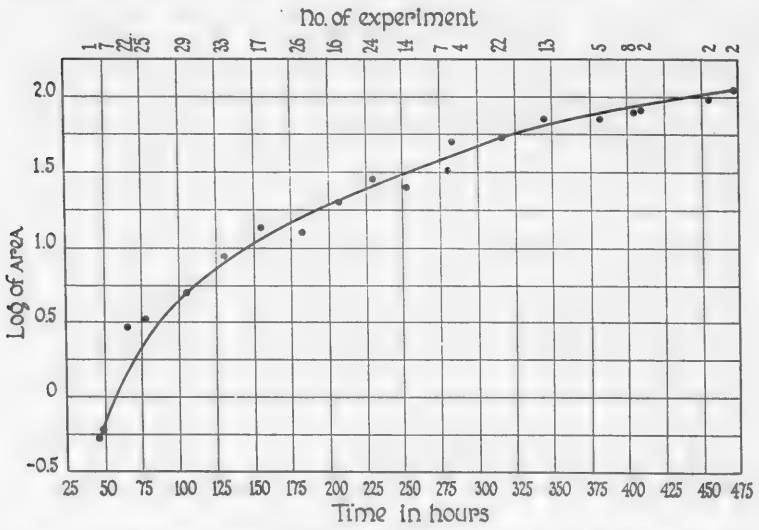


FIG. 2. This curve shows the increase of the projected area of the hearts with time. The method by which the measurements were made is described in the text.

logarithms of the heart rates have been plotted against the time of incubation (Fig. 3). The curve which it has been possible to draw begins at 50 hours (2 days, 2 hours) and extends to 450 hours (18 days, 18 hours). Its lowest point is at the beginning. It then rises sharply to about 140 hours (5 days, 20 hours) and continues to rise until the period btween the 15th and the 16th day; that is to say, to 251 beats per minute (log = 2.40). In the last 2 or 3 days of incubation the rate falls slightly (from 251 to 222).

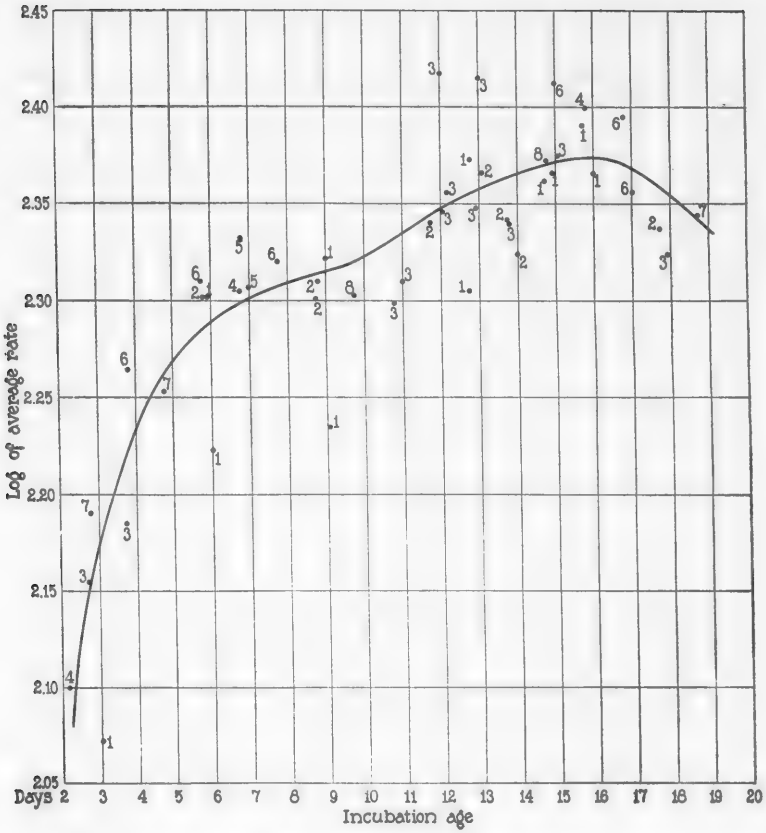


FIG. 3. A curve is shown which serves to indicate that in the course of incubation changes in the rate of the heart beat take place which appear to be of an orderly nature although the deviations from the average are large. The numbers accompanying the points indicate the number of experiments.

DISCUSSION AND SUMMARY.

These experiments show, then, that there are changes in rate which take place during the incubation period and that the direction of the curve expressing them changes in an orderly fashion. No attempt has yet been made to ascertain the influences operative in bringing

about these alterations. It is our intention, however, to attempt to correlate them with such processes as changes in the rate of the general metabolism of the body, changes in structure of the heart muscle, and if possible with changes in the inherent dynamics of the muscle.

It will be seen that the number of experiments at a given age is relatively speaking small. The temperature in spite of the precaution mentioned has likewise not been uniform, the range in the greatest number (38) of experiments having been 38°C. ± 1°. A few (four) counts have been made when the temperature was as low as 33.6°C. and a few (two) also at a temperature as high as 40°C.

Although a curve as smooth as the one which has been drawn in all probability represents the general course of heart rate change, an explanation of the deviations which have been found is desirable. At least two influences which may be operative suggest themselves. First may be considered the effect of the unavoidable injury incident to cutting a window in the shell and removing so much of the shell membrane as to make the object visible. It has been a uniform practise to discard those eggs in which hemorrhage took place, so that if opening the egg has a share in the difficulty, this operation may be thought of as exerting a mechanical stimulus on a nervous or other controlling mechanism. But whether, especially at the early ages, a mechanism of such a nature exists within the embryo, we do not know. The search for one lies for the present beyond the range of our interest.

A second influence responsible for the relatively large deviation in rate from the average may be that of fluctuations in temperature. The air of the room in which the observations were made was, in a given situation, fairly uniform at 38°C. ± 1.0°. as the temperature records show. In the earlier experiments the temperature was read at a distance about 1 foot away from the egg. Between this point and the egg a difference unknown to us may well have existed. It is improbable, however, that it can have been more than 2°C., that is to say, 38° ± 2°. Roughly if the change in rate is approximately 15 beats per degree a maximum deviation of 30 beats may be expected. In point of fact a deviation as great as this did not take place. In later experiments the temperature was read in the immediate vicinity of the eggs, so that a correction can be made more accurately.

There is in all probability a high degree of uniformity in the rate of the heart of the chicken embryo at each stage of development, although variation is also found. What the extent of the deviation is that may properly be encountered we do not yet know. It is desirable to pursue this subject further. Observations will accordingly be made and will be reported later.

CONCLUSION.

The study here reported has made possible the presentation of a curve which shows distinctly that changes take place in the rate of the heart beat during the development of the chick embryo.

BIBLIOGRAPHY.

1. Wernicke, R., Zur Physiologie des embryonalen Herzens, in *Samml. physiol. Abhandl.*, Jena, 1876, series 1, No. 5.
2. Murray, H. A., Jr., *J. Gen. Physiol.*, 1925–26, ix, 1.
3. Murray, H. A., Jr., *J. Gen. Physiol.*, 1925–26, ix, 39.

PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

VI. Differentiation in the Chicken Embryo Heart from the Point of View of Stimulus Production.

By ALFRED E. COHN, M.D.

With the Assistance of Edith L. Wile.*

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 2, 1925.)

This study concerns itself with the attempt to discover throughout the period of embryonic life of the chick, first, the site in the heart at which the pace-making function resides, and, second, to what extent other regions in the heart either share in this function or fail to share in it; to learn at what ages the various portions lose this capability in whole or in part. Studies of the functions of the embryonic chick heart have before now been undertaken, notably with the view to studying such functions as the conduction of stimuli (Külbs (1), Wertheim-Salomonson (2), Johnstone (3)) and to a less extent that of rhythm production and of reflex irritability (Johnstone). The observation that isolated fragments of the heart muscle such as were used in these experiments contract over long periods of time in appropriate culture media has been made by Carrel (4) and by Burrows (5). The object in the observations now reported has been to learn the extent to which a given portion of the living heart possesses this function.

Method.

After the rate of the heart of the intact embryo had been ascertained in the manner described in another paper (6), the embryo was weighed, the weight found being utilized to aid in establishing its age. The heart was then excised and its area measured (6). The heart was next dissected while still suspended and bathed

* We are indebted to Miss Alma Rosenthal for aid in the early part of these experiments.

in chicken serum. The fragments were planted in a large drop of chicken plasma to which enough chicken embryo extract was added to cause coagulation. So far the method of planting and the preparation of the materials resembled closely that for which we are indebted to Carrel. The modifications of his admirable method for our purposes has been described elsewhere (7). For this particular purpose a further modification was adopted in the choice of the embryo extract. Usually the extract had been that of embryos 10 days old. Here we chose instead embryos of the same age as that of the heart to be tested, with the view to exposing the fragments of the heart only to such tissue juices as were at the same stage of development. So far as the source of the plasma was concerned, we had no choice; even if it were obtainable, enough was obviously not available from embryos of the age in which we were most interested. It may be mentioned that the coagulation of the plasma after the fragments had been planted in it varied with the age of the tissue extract that was used. With extracts made from embryos before the age of approximately 5 days, the time of coagulation was delayed; afterward with increasing age of the extract, coagulation was more prompt. The dissections were carried out, as has been said, on the stage of a dissecting microscope, clean incisions being made by carefully sharpened iridectomy knives. After the fragments were planted and the medium coagulated the mica slide on which the plasma had been spread was inverted and placed on a Gabritschewski dish, in the depressed portion about the circumference of which water was placed to prevent drying of the preparation. The average time from the beginning of weighing to the completion of coagulation averaged 22 minutes, increasing with the increasing age of the embryo. At each step of the preparation care was taken to prevent evaporation as a result of the heat of the room and its low percentage of humidity.

We were, it seems, justified in our methods as the differences in behavior exhibited by the various fragments now to be described will show.

EXPERIMENTS.

Experiments were made on the hearts of embryos from 2 days, 18 hours old to 19 days, 18 hours old.

In the 2 to 3 day embryos, when the heart is still in the stage of a simple tube, before the development of the interauricular septum, the whole heart was divided into four pieces (Fig. 1), the atrium, the proximal and distal parts of the ventricle, and bulbus arteriosus. Later at about the 3rd to the 4th day (Fig. 2) the atrium was divided into two pieces, right and left; and the ventricle into five portions, two of the right ventricle, two of the left, and an apical portion. Later still from about the 7th day onward, the auricle was divided into five portions, a mesial piece, two pieces of the right auricle and two of the left (Fig. 3). When the auricles

were large so that the fragments measured more than 1.0 sq. mm., each of the standard five auricular fragments was further subdivided. In this manner, throughout the experiments, the whole auricular tissue was fractionated, planted, and the rate developed by each fragment counted and recorded.

The treatment of the ventricles differed from this. As soon as the heart was large enough, at about the 7th day, these were divided into seven instead of five pieces, —two each from the right and left ventricles, and one each from the apex, conus arteriosus, and the base of the ventricles behind. The pieces from the ventricles

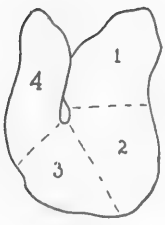


FIG. 1.

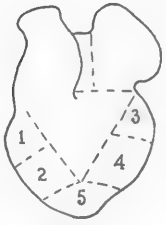


FIG. 2.

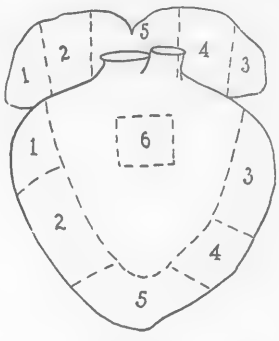


FIG. 3.

FIG. 1. An outline drawing is shown of the heart of a chick embryo 2.7 days old. The heart was divided in a manner indicated by the interrupted line. The portion numbered 1 is the venous end of the tube; that numbered 4, the arterial end.

FIG. 2. In this outline drawing of the heart of a chick embryo 3.9 days old, the manner is shown in which the auricles were divided. The portions numbered 1 and 2 represent the locations of the wall of the right ventricle from which pieces were taken; 3 and 4, those from the left ventricle; 5 that of the apex.

FIG. 3. An outline drawing is shown of the heart of a chick embryo 7.7 days old. The number and location of the fragments of the auricles as well as of the ventricles are shown. Fragment 6 was taken from the conus arteriosus. An additional piece, 7, was taken from the base of the right ventricle posteriorly.

and apex were taken from the margins, right and left, as representative of the convex border of the primitive tube. Except for the conus and dorsal pieces, the V-shaped area between the right and left borders (Fig. 3), including the septum, was discarded. Later when the heart was larger still, seven fragments continued to be taken, but the five pieces from the margins of the heart, instead of being adjoining portions, were specimens only of the five regions. In these large hearts, therefore, only a small portion of the total ventricular tissue was studied.

In the earlier experiments (Nos. 9 to 60) all the fragments were projected and measured according to a technique described elsewhere (7), and the measurements correlated with the rate developed by the fragment. Studies of this sort were made of fragments beginning at 3 days, 18 hours old and continued to 11 days, 18 hours. The measurements ranged from 0.05 to 0.86 sq. mm.; the great majority between 0.1 and 0.25 sq. mm. When the size of the fragments containing the pace maker taken from hearts of various ages is studied in relation to the rate developed by each, the absence of correlation between size and rate quickly appears (Fig. 4). In 4 day embryos the

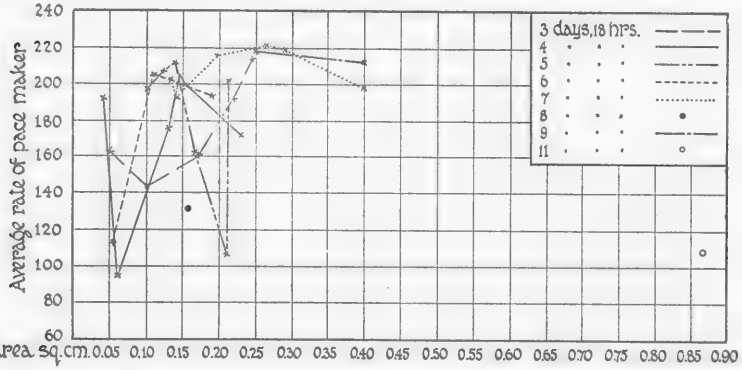


FIG. 4. A series of curves is shown to demonstrate the fact that the size of the fragments of the pace maker makes no difference in its rate-making capability.

smallest pace maker exhibited the highest rate; in 3 day embryos it was the largest piece which did so; in a 7 day embryo pieces ranging from 0.13 to 0.4 sq. mm. all beat at a high rate, from 192 to 220 per minute.

The rate of each fragment was counted as soon after planting as possible. The counting continued for about $1\frac{1}{4}$ hours; in the later ones for 1 hour. In many, indeed in most, experiments the counts after the first 10 minutes of planting were surprisingly constant so that the deviation from the average rate of a given fragment was less than 10 beats in 58.7 per cent of the cases and not more than 30 beats in 94 per cent of the cases (Table I). Obviously therefore the rate of each fragment shortly after planting was approximately uniform.

In another paper (6), the rate of the heart of the intact embryo of various ages is reported and the fact is pointed out that a curve can be drawn which shows the general direction of changes in rate during embryonic life. It is likewise pointed out that, for reasons not yet clear, considerable deviations occur. The specimens included in that study formed the basis also for this one. It is not surprising, therefore, that at a given age the fragments containing the pace maker developed different rates. That the differences are greater than were found in the intact embryos was to be expected, because of the highly artificial environment in which the fragments were placed. In view of this circumstance and of the fact, moreover, that not so much the actual rates developed but the rate of the pace maker relative to that

TABLE I.

Deviation of Each Fragment from Its Average Rate.

No. of fragments.	Deviation from average rate.	Average per cent for average rate.	Per cent of fragments.
Total....577	0–209 beats.		100
46	0	0	8.0
110	1–3	10.7	19.0
183	4–9	18.3	31.7
	Below 10.		58.7
205	10–30	37.3	35.6
33	Above 30.	76.6	5.7

of other fragments can have physiological significance, an analysis of the actual rates has been neglected. It is, however, of interest to notice (Table II) that of 88 instances studied, the difference in rate between the intact heart and that of the fragment containing the pace maker was 3.5 per cent or less in 21 (= 24 per cent) cases; 5.3 per cent or less in 37 (= 42 per cent) cases; and 6.7 per cent or less in 48 (= 55 per cent) cases. The close approximation of the two rates is a striking and surprising circumstance, for we have now a demonstration that the size of the fragment makes no difference to rate production, and that the fragments can actually equal and sometimes surpass the rate of the intact heart. This occurred in point of fact in 28 experiments.

TABLE II.

The Rate of the Pace Maker Compared with That of the Whole Heart.

Age.	No. of cases in which rate of pace maker and of whole heart differ by.																																				
	0–10				10–20				20–30				30–40				40–50				50–60				60–70				70–80				80–90				90–100			
	Whole heart higher.		Pace maker higher		Whole heart higher.		Pace maker higher.		Whole heart higher.		Pace maker higher.		Whole heart higher.		Pace maker higher.		Whole heart higher.		Pace maker higher.		Whole heart higher.		Pace maker higher.		Whole heart higher.		Pace maker higher.		Whole heart higher.		Pace maker higher.		Whole heart higher.		Pace maker higher.		Whole heart higher.		Pace maker higher.	
days *hrs.*																																					
2 18*			1			1				1				1							1						1								2			
3 18	2		1							1														1													
4 18	2					1		1	1	1				1													1										
5 18	2			1		1		1	1	1			1								1				1												
6 18	2		1	1												1									2				1							
7 18			1	1		1		1	1						1																						
8 18			2	1									1														2										
9 18			2	3		1											1																				
10 18	1					1				1				1																							
11 18																																					
12 18	1			1																																	
13 18				1																	1	1															
14 18	2		1						1				1																								
15 18																									1												
16 18				1																	1																
17 18									1	1																											
18 18																																					
19 18																											1										
Total....	12		9		10		6		5		6		3		4		2				4		1		4		1		6					2			

Age.	100–150				150–200				200–250				Total.
	Whole heart higher.		Pace maker higher.		Whole heart higher.		Pace maker higher.		Whole heart higher.		Pace maker higher.		
days *hrs.*													
2 18*	2												10
3 18													5
4 18													7
5 18													9
6 18													8
7 18	1												6
8 18													6
9 18	2												9
10 18	2												6
11 18	1												1
12 18													2
13 18													3
14 18													5
15 18									1				2
16 18	1								1				4
17 18	1												3
18 18	1												1
19 18													1
Total....	11								2				

*18 hours was the average time, varying ± 3 hours.

The Location of the Pace Maker.

The venous end of the cardiac tube was divided in the manner already described, depending on the age of the embryo, the incisions after the 3rd day being made parallel to the long axis of the organ.

TABLE III.

Location of Pace Maker.

Age.	No. of cases.	Right auricle.	Middle piece.	Left auricle.	Ventricle.				Remarks.
					Right base.	Proximal portion.	Distal portion.	Bulbus arteriosus.	
days hrs.		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
2 18*	10	40†				20	20	20	Ventricular portions were equal in rate with the venous end.
3 18	6	50	33			17			
4 18	7	62.5	37.5						
5 18	9		77	22					
6 18	7	13	87						
7 18	6	16⅔	66⅔	16⅔					
8 18	6	83	16						
9 18	8	37.5	37.5		25‡				
10 18	5	80	20						
11 18	1								
12 18	3	100							
13 18	3	66⅔	33⅓						
14 18	5	100							
15 18	2	100							
16 18	4	75		25					
17 18	3	33⅓	33⅓	33⅓					
18 18	2	50	50						
19 18	2	100							

* 18 hours was the average time, varying ± 3 hours.

† At this age there is included the whole venous end of the tube.

‡ To these pieces part of the right auricle was probably attached.

In embryos 2 days, 18 hours old a sharp differentiation of function has apparently not yet taken place. Fragments from the venous end usually (in 40 per cent of cases) set the pace, but this function can at this age be assumed also by the two ventricular and the arterial fragments (Table III). The inference cannot be drawn from these facts

that in the intact heart under natural conditions there is indifference in respect to which portion of the tube initiates the pace. The experiments permit the statement only that at this stage of development a high rate can be developed by fragments from any part of the tube; that the tube in short does not yet show a notable degree of differentiation. At 3 days, 18 hours, a change has already taken place. In the majority of the cases, the rate-making functions appear to lie in the auricle, whereas the ventricles appear to retain the function occasionally. It must be remembered, however, that there is a practical difficulty encountered in dividing the heart. Theoretically if the incision passes proximal to the line of junction between auricle and ventricle, even to a slight extent, a few auricular fibers or cells possessing a high inherent rate if joined to a ventricular fragment may set the pace and give to the ventricles the appearance of possessing a capability which is not inherent in them (Table III). Actually in these experiments at the earlier stages of development the behavior of the fragments anterior to the position of the atrioventricular line shows that they are perhaps the most sensitive.

After the 3 day, 18 hour period, there is no doubt that one or other of the auricular fragments takes on the leadership in rate making. At this time it was usual to divide the auricles approximately into a right portion (usually sub-divided into two fragments), into a left portion (likewise usually divided into two fragments), and into a middle piece at the location of the septum. Obviously it was not possible to separate these structures in such a manner that the middle piece retained in each instance precisely the same structures as the right and left portions. If the pace-making function resided in the mesial portion of the right auricle, it became a matter of chance, depending on the location of the incision, as to whether the pace was to be set by the right or the middle. Occasionally the incision was misplaced sufficiently so that pace making appeared to reside even in the left fragment. In the younger embryos, from the 5th to the 7th day, it was usual to include the pace maker in the middle or right piece. In the larger embryos, where incisions could be placed with greater accuracy, it was the right piece which set the pace. In any case it was not the laterally placed fragments (tip of auricles), but the

mesially placed ones which contained the rate-making structure (Table III).

What the histology of this portion of the auricle is we have not investigated in detail. We know, however, from the study of series of chicken embryos examined at the Harvard Medical School[1] that nerve ganglia are located in the right auricle, in the posterior wall near the interauricular septum. Precisely what function these elements exert we do not know. From the manner, however, in which the incisions were placed, there is no reason to doubt that they were included in the fragment of auricular tissue which usually set the pace. Their presence here throws no light on the question of which tissue, nervous or muscular, is responsible for the cardiac rhythm. A rhythm exists before the inwandering of ganglion cells on the 5th to the 6th days, as is well known since the studies of His (8). What relation to this problem the controversy (Kunz) on the problem whether ganglia wander first into the heart, trailing their nervous connections behind them, or whether the nerve fibrils penetrate the heart tissue first, and the ganglion cells later, is not known. A decision is difficult because there is as yet no adequate method for staining the nerve fibrils and of tracing their course in the heart.

The change in function of rhythm production in fragments taken from typical positions in the heart was studied from the point of view of differentiation. By rhythm production is meant the ability to contract under the experimental conditions, as distinguished from the function of pace making by which is meant the ability to contract at a highest rate in a particular heart. Some fragments contracted; others failed entirely to do so. It is, of course, clear that statements concerning the fragments treated in the manner adopted in the experiments are not generally valid. It is wholly conceivable that the same fragments, had they been suspended in one or another balanced salt solution, might very well have contracted. The problem here was, however, to distinguish diversities if they existed, not uniformity.

[1] It is a great pleasure to be able to acknowledge the courtesy shown to us by Professor F. T. Lewis in placing the beautiful series in his department at our disposal for study, and to thank him for the opportunity he afforded us. To Professor J. L. Bremer and to Dr. E. A. Boyden we are likewise indebted for aid and suggestions.

TABLE IV.

*The Loss of Rhythmic Function by the Fragments According to Age except the Pace Maker.**

Age.		No. of cases.	Auricles.						Ventricles.							
			Right.			Left.			Right.		Left.		Apex.	Arterial end of tube.	Conus arteriosus.	Base of the ventricles posteriorly.
			1	2	3	3	2	1	Base (proximal).	Mid level.	Base (distal).	Mid level.				
days	*hrs.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2	18†	9	57					100	88		88			33		
3	18	6	100					100	100		100	100		66		
4	18	7	100	100‡	100	100		100	100	100	100	100	50			
5	18	9	100		100	88	100	80	100	100	100	100	100			
6	18	7	100	100	100	100	100	100	100	100	100	100	100		100 (1)§	
7	18	6	100		100	100		100	100	100	100	100	100		100 (1)§ (root of aorta).	
8	18	6	100		100	100		100	100	80	40	80	80		66 (3)§	100 (2)§
9	18	8	100		100	100		100	100	100	100	100	100			
10	18	5	40		60	80	100	80	60	60	60	20	80		20	0
11	18	1	100		100	100		100	0	0	100	0	0		0	0
12	18	3	33	33	100	66	66	33	66	66	66	66	0		0	0
13	18	3	0	33	66	33	33	0	0	0	33	33	33		0	0
14	18	5	0	40	100	20	0	0	0	0	0	0	0		0	0
15	18	2	0	100	0	0	0	0	0	0	0	0	0		0	0
16	18	4	25	33	50	50	0	0	0	0	0	0	0		0	0
17	18	3	0	33	33	50	0	33	0	0	0	0	0		0	0
18	18	2	0	50	50	0	50	0	0	0	0	0	0		0	0
19	18	2	0	0	100	0	0	0	0	0	0	0	0		0	0

* The pace maker contracted, of course, in 100 per cent of the cases.

† The age of the embryos included varied between 17 and 22 hours or 18 ± 4.

‡ Occasionally the auricles were each divided into three pieces. The mesially placed piece in each case is then numbered 3. This method of division is not shown in Fig. 3.

§ The numbers in parentheses are given only when all of the cases at the given age did not have fragments of the particular location mentioned—and to indicate the number of fragments studied.

The choice of the method we employed proved fortunate in point of fact for it furnished the opportunity of bringing out differences.

In the experiments in the 2 and 3 day embryos the distal, arterial end of the tube has clearly lost the spontaneous contractile function in one- to two-thirds of the cases (Table IV). All the other fragments no matter whence taken retain this function until the 7th day. On the 8th day the ventricles begin to exhibit a loss of the function and by the 13th day this is complete. On the 10th day the auricles, except of course the pace maker, that is to say the lateral fragments especially, also begin to fail. There is, in short, as embryonic life draws to a close apparently a continuous diminution of the capability of independent contraction until at the end it is lost almost entirely, with the exception always of the area to which rhythm production is ultimately confined.

DISCUSSION AND SUMMARY.

In these experiments we have shown that, with the technique adopted, differences in behavior are exhibited by fragments of the heart taken from different localities. The different localities behave in a more or less uniform manner. The pace-making function, for instance, is found at first throughout the cardiac tube but later it is restricted and comes to reside in a special small area at the back of the right auricle near the center. The pace-making system is able to develop a rate comparable to that shown by the whole intact heart, irrespective of the size of the fragment in which it is contained. Later, under the circumstances of the study, the ventricular structures lose the power of spontaneous contraction, and later still, the auricular ones also. It need scarcely be pointed out, however, that this loss refers only to the function of pace making. In its place, the various localities of the heart undoubtedly take on other capabilities. This is what is meant after all by differentiation.

The question whether the pace-making and conduction systems reside in the remains of primitive portions of the cardiac tube in an undifferentiated form, or whether on the other hand these primitive portions develop into differentiated structures which preside over these functions may be reviewed afresh. Obviously the tube in its early state possesses these functions; obviously also the major part of the

heart loses them during the course of development. A knowledge of the changes in form paralleling changes in function would have great interest. On this phase of the problem we hope to report later.

On the basis of these observations, differentiation from the point of view of stimulus production may be viewed perhaps in this manner. Pace making, the conduction of impulses, and contraction are the primitive functions of the tube. As the tube develops into the adult structure, pace making and conduction are supposedly served by tissues resembling in structure the original ones. Whether as a matter of fact a structural change takes place is an interesting and important problem. Those portions of the heart which require to develop greater degrees of energy lose the primitive functions of pace making and conduction, and, in the transformation, take on a differentiated structure. It is, then, not the structures in which the primitive functions of pace making and conduction reside which are differentiated, but the greater mass of ventricular muscle. These reflections have their origin not only from our own work but they grow out of observations to be found in the writings of those (A. Keith and I. McKenzie) who call the nodal and conduction tissues in the heart, embryonic. But whether from the point of view developed here the use of this term is completely descriptive remains an interesting problem.

BIBLIOGRAPHY.

1. Külbs, F., in Cramer, M., Beiträge zur Physiologie, Berlin, 1914–20, i, 439.
2. Wertheim-Salomonson, J. K. A., *Arch. ges. Physiol.*, 1913, cliii, 553.
3. Johnstone, P. N., *Bull. Johns Hopkins. Hosp.*, 1924, xxv, 87.
4. Carrel, A., *J. Exp. Med.*, 1912, xv, 516.
5. Burrows, M. T., *Science*, 1912, xxxvi, 90.
6. Cohn, A. E., *J. Exp. Med.*, 1925, xlii, 291.
7. Cohn, A. E., and Murray, H. A., Jr., *J. Exp. Med.*, 1925, xlii, 275.
8. His, W., Jr., *Arb. med. Klin. Leipzig*, 1893, 14.

STUDIES ON THE ANTIGENIC SUBSTANCE OF THE BACTERIAL CELL.

By HANS ZINSSER, M.D., AND TAKEO TAMIYA, M.D.

(From the Department of Bacteriology and Immunology of Harvard University Medical School, Boston.)

(Received for publication, May 16, 1925.)

In the course of a number of years during which we have occupied ourselves with the study of bacterial antigens, we have become convinced that the progress of immunological knowledge has been not inconsiderably impeded by the tendency to apply all the facts ascertained by the study of coagulable proteins, such as horse serum, egg white, etc., by direct analogy to the reactions induced in animals infected or treated with bacteria. Our earlier studies (1) on the differentiation between the bacterial allergies, such as the tuberculin reaction and protein anaphylaxis, persuaded us that we could not hope to make consistent progress in the further understanding of the biology of bacterial infection and immunity unless we could obtain more precise knowledge concerning the bacterial antigenic substances which, in quite obvious chemical properties, differed from the antigenic true proteins.

Our studies with Parker since that time (2), and those of a number of our associates, have occupied themselves with a variety of bacteria, including tubercle bacilli, pneumococci, meningococci, staphylococci, and typhoid, colon, and influenza bacilli, and our associate, Mueller (3), has carried out special studies with yeast and, with Smith and Litarczek, with bacilli of the Friedländer group. These investigations have yielded results that are, in certain fundamental aspects, consistent among themselves and, in large part, in harmony with the very important studies carried on by Avery and Heidelberger (4) along closely related problems.

The conditions may be summarized at the present time, briefly, as follows:

None of the bacteria studied so far contain any considerable amount of heat-coagulable albumin or globulin. These materials are probably always present to a slight extent in bacterial extracts and may determine the limited formation of antiprotein antibodies and, consequently, of true anaphylaxis. But they do not represent the predominant antigenic constituents of the bacterial cell.

Occasionally, small amounts of a substance acting like Bence-Jones protein has been found, especially in extracts of tubercle bacilli, but this, when present, is small in amount and of undetermined significance, though, as Bayne-Jones (5), Hektoen and Welker (6), and others have shown, this, too, may form its own homologous antibodies.

All the bacteria investigated have yielded considerable amounts of two substances which have seemed to be of particular importance, and which differ, both chemically and in their biological attributes, from the antigenic constituents of animal blood serum and perhaps of tissues.

One of these is the material which we have referred to in a number of papers as the "residue substance," and which we believe to be identical with the biuret-free, precipitable, but not antigenic, digests of typhoid extracts described by Pick as early as 1912 (7), surely the same as the "soluble" pneumococcus substance observed by Dochez and Avery (8) in the urine and blood of pneumonia patients and in the filtrates of broth cultures. This material may be obtained by a variety of methods from all bacteria so far investigated by us. It is diffused out into fluid culture media; it may be extracted from the ground or unground bacterial sediments, either by simple extraction or after moderate antiformin treatment; and from some bacteria, notably pneumococci, meningococci, and influenza bacilli, it may be easily washed off the bacterial surfaces by brief shaking in neutral isotonic solvents. It is relatively heat-stable; gives no protein reactions; is alcohol-precipitable; and, according to the precise chemical studies on pneumococcus made by Heidelberger and Avery (9), with which the investigations of Mueller on yeast and tubercle bacilli (10) agree, their structure is that of a complex carbohydrate.

Biologically these substances are precipitable, give complement fixation reactions with homologous antibodies, and are specific—so delicately group-specific, indeed, that there seems little doubt about their representing the particular material upon which this property of the whole bacteria depends. In the form in which they are obtained separate from the bacteria, however, they are completely incapable of inducing antibody formation. The ease of their removal in the case of capsulated organisms has quite obviously suggested to us, as it has to Avery, their possible morphological involvement with capsular material, a thought which is strengthened by the possibility that their capacity to unite with, *i.e.* divert, antibodies outside the living cellular structures may explain the insulating protective functions of the bacterial capsule.

The second material which has invariably been obtained from all bacterial extracts, and in quantities which have seemed to determine it as the bulk of the extractable bacterial substance, is of a protein nature, since it gives qualita-

tive protein reactions, but was designated by us in our first paper as "nucleoprotein," largely because it is precipitable by acids in the cold, at quite definite pH ranges, redissolving at neutrality and slight alkalinity, and in excess of acid. The exact chemical definition of this material must await the collection of sufficient amounts for systematic analysis—a task now being undertaken by Mueller and his associates in this laboratory, but it is quite obviously not an ordinary coagulable protein, since, if boiled in acid suspension while it is flaking out, these flakes immediately redissolve when the solvent is neutralized to pH 6.9 to 7.2.

This last material is also antigenic, not as strictly specific as the residue,[1] but capable under certain conditions not only of reacting *in vitro* with antibodies, but also of inducing antibody formation when injected into animals. As studied so far, the nucleoprotein is probably a mixture of a number of substances not functionally separable up to the present time. The various biological properties we attribute to it, therefore, must be regarded for the present as those of the impure substance which may, on further study, be again divisable into a number of functional parts. Thus, we have already described, as a result of the studies of Mueller and those of Mueller with one of us (11), that the nucleoprotein fraction of tuberculin and tubercle bacillus extracts seems to carry down with it in an impure condition the material responsible for the tuberculin reaction, and in this function is entirely separable from the residue. This is being further investigated at the present time by Mueller.

In our first study in 1921 on the tuberculin reaction, in which the separation of the bacterial substances into the two fractions noted above was carried out, we suggested the possibility that the nucleoprotein might represent "the mother substance from which the residue material was derived." Later, in studying the antibody reactions and confirming our earlier failures to obtain antibodies by injection of residue materials in spite of prolonged efforts, we mentioned the possibility that the residue material constituted what Landsteiner (12) had spoken of as "haptenes" and since that time the extreme specificity of these materials, together with their impotence to incite antibodies, has led us to speak of them as the "haptophore group" of the bacterial cell. We suggested at that time that molecular size and anti-

[1] We do not include experiments on the specificity of nucleoprotein antibody reactions in the text in the interests of brevity. Cross-reactions with nucleoproteins and antisera of streptococcus, pneumococcus, and tubercle bacilli showed group reactions with the concentrated material between streptococcus and pneumococcus nucleoproteins, union with either of them and the tubercle bacillus antiserum of group reactions, however, being eliminated in dilutions of 1:10. In connection with this see, also, the work of Lancefield.

body formation might be related, the residue material representing the haptophore group that had been split off from the larger molecule, union with which had made it a complete antigen. We tried at that time to obtain residue material by subjecting nucleoprotein to digestion in the autoclave at various hydrogen ion concentrations and various pressures, and last year attempted a similar experiment with horse serum and egg albumin, believing that we might be able to split off such a haptophore group from these materials, but also without success (13).

In the experiments presented in the present paper we have attempted to obtain a better understanding of the relationship between these antigenic fractions of the bacterial cell by immunizing a series of animals with whole bacteria and nucleoproteins obtained from tubercle bacilli, streptococci, and pneumococci and comparatively studying the antibodies obtained as a result.

As will be seen in the protocols in the very simple experimental procedures recounted below, the materials used throughout were whole bacteria, undissolved and dissolved, and nucleoproteins produced by the ordinary methods and employed both unfiltered and after filtration through Berkefeld filters for the removal of all bacterial fragments.

Experiment 1. Tubercle Bacilli.

February 4, 1925. 50 cc. of ten times concentrated synthetic bacillus broth precipitated with $\frac{N}{1}$ HCl. Centrifugalized. Precipitated nucleoprotein taken up in 50 cc. salt solution at pH 7±. Supernatant fluid precipitated by the fractional alcohol precipitation of Mueller. Two volumes of 95 per cent alcohol added. Centrifugalized. Precipitate discarded. Three further volumes (volume counted as original volume of material) now added to the supernatant fluid. Centrifugalized and redissolved in 50 cc. of salt solution. We now have three substances for test:

1. The original whole material.

2. The nucleoprotein precipitate from 50 cc. again dissolved in 50 cc. of salt solution and therefore representing probably somewhat less than the original concentration of this material in the whole broth.

3. The residue material precipitated by fractional alcohol precipitation redissolved in 50 cc. of salt solution and likewise, allowing for loss, representing somewhat less than the original concentration of this material in the whole broth.

Precipitations with this material were now done with two sera:

1. Serum A, obtained from a rabbit immunized with living H 37 tubercle bacilli.

2. Serum B, obtained from a rabbit immunized systematically with unfiltered nucleoprotein.

Results of precipitations were as follows:

	1			2		
	Serum A—living H 37 tubercle bacilli.			Serum B—nucleoprotein.		
Dilution.	Whole material.	Nucleo-protein.	Residue.	Whole material.	Nucleo-protein.	Residue.
Concentrated.	−	++ to +++	−	−	−	−
1:5	+++	+	+++	+++	+++	+++
1:10	+++	±	+++	+++	+++	+++
1:20	+++	−	+++	+++	+++	+++
1:40	+++	−	+++	+++	+++	+++
1:80	+++	−	+++	+++	+++	+++
1:100		−	++	++ to +++	++	++
1:200			+	+	+	+ to +++
1:300			+	−	±	−
1:400			±	−	±	−
1:500			−	−	−	−

It will be seen from this experiment that the antiserum produced with whole tubercle bacilli had little precipitating power for the nucleoprotein material; whereas that produced by the injection of nucleoprotein material precipitated both the residue and the nucleoprotein.

This relationship could not, however, be accepted as a conclusive definition of the true facts, since the nucleoprotein material with which Rabbit B (2) had been treated had not been filtered, and it was possible that the activity of its serum upon residue might have been due to the bacilli and bacillary fragments undoubtedly present in the nucleoprotein in spite of repeated resolution and reprecipitation. We therefore repeated this work with filtered nucleoprotein, made just as before from alkaline extracts of tubercle bacilli and passed through Berkefeld candles. In doing this work we have not been

able to explain why it has been so exceedingly difficult to induce any kind of antibody formation with the filtered nucleoprotein of the tubercle bacillus. This, however, is the case and it was only by persistent effort and the injection of large amounts of the filtered material that the results recorded in Experiment 2 were finally obtained.

This experiment shows that when the nucleoprotein is filtered free of any traces of bacterial fragments no antibody to the residue is formed.

Experiment 2. Tubercle Bacilli.

Comparison of Antibodies in Sera Prepared with Unfiltered and with Filtered Nucleoprotein Respectively versus Tubercle Bacillus Nucleoprotein and Residue.

	Serum C (nucleoprotein unfiltered).	Serum D (nucleoprotein filtered).	Serum E (nucleoprotein filtered).
Nucleoprotein.			
1:100			
1:250	++++	+++	+++
1:500	++++	++ to +++	++
1:1000	++	+	±
	+	±	±
Residue.			
Concentrated.	+++	±	−
1:5	+++	−	−
1:10	+++	−	−

With streptococci similar conditions appear to prevail, as is apparent from the following experiment which was performed incidental to streptococcus immunizations done with the Dick strains of scarlatinal origin, at this time, by one of the writers with Dr. F. B. Grinnell.

Experiment 3. Hemolytic Streptococci.

Rabbit F, immunized with whole streptococci sediment from broth cultures.
Rabbit G, immunized with streptococcus nucleoprotein.

Precipitations.

	Streptococcus Serum F (whole bacteria).	Streptococcus Serum G (nucleoprotein serum).
Nucleoprotein.		
Concentrated.	++++	++ slow.
1:5	+++	++
1:10	+++	+
1:50	+++	±
1:100	+	−
Residue.		
Concentrated.	++++	−
1:5	++	−
1:10	+	−
1:50	−	−

Here, again, it is apparent that while the whole bacteria induce antibodies both to the nucleoproteins and to residue, immunization with filtered nucleoproteins fails to induce any antibodies which react with the residue material.

It thus appears, from the foregoing, that when the bacterial body is extracted by the relatively gentle method of treatment with a weak alkali, and this extract then fractionated by the acid precipitation method, two substances are obtained—one the nucleoprotein which seems to be independently antigenic, inducing antibodies which react only with itself and not with the residue; the other the residue material incapable of inducing any kind of an antibody reaction, but capable of reacting with antibodies formed by injection of the whole bacteria. It would seem, from this, that the residue represented the haptophore group of something left behind in the bacterial bodies during the extraction. Since it is obviously quite without promise to approach closer to a solution of this problem by immunizing with the surely complex mixture of the sediment remaining after the extractions, we determined to try the following, using pneumococci largely because of the ease with which these organisms can be dissolved in bile:

1. To immunize a rabbit with intact, undissolved pneumococci.

2. To immunize another animal with all the materials obtained in pneumococcus solutions after filtration to remove all formed elements.

The two animals would thus, to all intents and purposes, receive the

total pneumococcus substances—in the one case, however, intact and morphologically complete—in the other, after solution.

It is, of course, impossible to devise methods of doing this which, on the one hand, can be held to avoid completely the injection of dissolved materials and, on the other, safeguard against the possibility of chemical change during solution. Conscious of this, however, we chose what we thought would represent the nearest approach to the desired conditions.

Experiment 4. Pneumococcus.

1. Pneumococci were grown on blood agar in pie plates. They were washed off with 2 per cent formalin solution, allowed to stand a few minutes, twice washed in salt solution, and immediately injected—intraperitoneally.

2. Similarly grown pneumococci were dissolved in the smallest amounts of ox bile which appeared to give complete solution, filtered through Berkefeld candles and similarly injected. A number of animals were lost in this process, probably because of the toxicity of the bile.

We did not drive these sera up very high, because the procedures were poorly tolerated by the animals and we did not wish to delay our work. Moreover, we believed that the basic principles could be demonstrated by relatively low titer sera.

	Serum H. Rabbit injected with formalinized pneumococci.	Serum I. Rabbit injected with bile-dissolved pneumococci.	New York State antipneumococcus serum.
Nucleoprotein.			
Concentrated.	+++	+++	
1:5	++	++	+++
1:10	++	++	+++
1:20	++	+	+++
1:50	++	±	++
1:100	+	±	++
1:200	+	−	+
1:400	−	−	±
Residue.			
Concentrated.	++	−	Not done.
1:5	++	−	" "
1:10	+	−	+++
1:20	+	−	++
1:50	−	−	+
1:100	−	−	−
1:200	−	−	−

SUMMARY.

Putting together the results of such experiments as those outlined above, we can set down the following definite facts.

1. The substance of the bacterial cell can be roughly divided into two antigenic entities. One of these is the so called "nucleoprotein" substance, the other the residue substance or soluble material of Dochez and Avery and Avery and Heidelberger, both of which have been repeatedly characterized in preceding papers.

2. Immunization with the nucleoprotein, if such nucleoprotein is rendered free of bacterial bodies or fragments of bacterial bodies by Berkefeld filtration, incites the production only of antinucleoprotein antibodies which, with slight group overlapping, are species-specific but, as determined by the previous studies of Avery and, subsequently, those of Lancefield, are not type-specific to the same degree as the residue antibodies.

3. Immunization with dissolved residue alone leads to no antibody formation whatever. This residue, as indicated in several of our own previous studies, represents the haptophore group upon which specificity depends and which, in the simple process of solution, is disrupted from another substance together with which it represented a complete antigen in the antibody-forming sense.

4. The formation of specific antiresidue antibodies is apparently dependent upon the injection of morphologically formed elements, at least as far as experiment can determine at the present time; for, as in the pneumococcus experiments, the most available process of solution and the injection of all the materials so obtained from the whole bacteria fails to yield antiresidue antibodies, as though in the mere process of dissolving the residue haptophore group were dissociated from its association with the larger molecule to which, in the whole bacteria, it lends specificity.

5. While antiresidue antibodies are only formed when such undisrupted bacterial cell substances are present in the immunizing substance, immunization with whole bacteria, even when attempts are made to preserve them from solution by formalin, leads to the formation of both antiresidue and antinucleoprotein antibodies, probably because a certain amount of solution inevitably takes place after injection within the animal body.

DISCUSSION.

In our earlier publications we assumed that the residue haptophore group is probably split off from the nucleoprotein, which may be regarded as its mother substance. Attempts to approach this by splitting residue off from nucleoprotein after it was obtained by the ordinary methods by digestion at various hydrogen ion concentrations, etc., were failures. As the matter stands at the present time, there are two possible interpretations. On the one hand, we may assume that the bacterial body contains two separate antigenic complexes—one concerned with the ectoplasmic capsule-forming zone in which the residue material, together with some protein substance, represents an antigenic union easily disrupted and separate from the nucleoprotein basic substance of the bacterial cell. This original residue-protein combination when split by solution would then yield two separate substances, neither of which is alone antigenic, and the residue recognizable later only by its ability to react with antibodies formed with material in which the two are still united and, therefore, antigenic. The only antigenic substance left in the dissolved material, then, would be the nucleoprotein, which would represent the second antigenic complex of the bacterial material constituting its bulk, and it is more cytoplasmic than specific in an entirely separate system from the one involving the residue. This would involve assuming that the second constituent of the original residue complex is entirely lost in experiments like the ones cited above.

A simpler explanation would be to return to our original interpretation, that the antigenic complex of the whole bacterial cell consists of a combination of nucleoprotein and residue material in which the residue determines the specificity of the total, just as in the work of Landsteiner certain methyl substances, etc., may alter the specificity of proteins to which they are attached. We are at the present time inclined to favor the latter position, first of all because it is simpler, and in the second place because when we inject the total dissolved pneumococci we get only antinucleoprotein antibodies. Moreover, it seems to be the antiresidue antibodies which determine agglutination and perhaps their reactions toward whole bacteria and antiserum, a subject which is still under investigation.

The only absolutely crucial experiment that could finally determine this matter in a simple way, would be success in synthetically reuniting residue with nucleoprotein. Since there seems no immediate hope of attaining this, we believe that indirect methods such as the one we have indicated above must be persisted in for the time being.

BIBLIOGRAPHY.

1. Zinsser, H., *J. Exp. Med.*, 1921, xxxiv, 495.
2. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275.
3. Mueller, J. H., and Tomcsik, J., *J. Exp. Med.*, 1924, xl, 343. Mueller, J. H., Smith, D. E., and Litarczek, S., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 373.
4. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 81.
5. Bayne-Jones, S., and Wilson, D. W., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 37.
6. Hektoen, L., and Welker, W. H., *J. Infect. Dis.*, 1924, xxxiv, 440.
7. Pick, E. P., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1912, i, 781.
8. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.
9. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73; 1924, xl, 301.
10. Mueller, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 209.
11. Zinsser, H., and Mueller, J. H., *J. Exp. Med.*, 1925, xli, 159.
12. Landsteiner, K., *Biochem. Z.*, 1918–19, xciii, 106.
13. Zinsser, H., *J. Immunol.*, 1924, ix, 227.

STUDIES ON THE ETIOLOGY OF JAGZIEKTE.

I. The Primary Lesions.*

By E. V. COWDRY.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 14 to 16.

(Received for publication, May 25, 1925.)

Jagziekte is usually regarded as a specific chronic catarrhal pneumonia of sheep which is peculiar to South Africa. The name is is derived from the Dutch *jagt*, to drive, and *ziekte*, a sickness, the combination of which is intended to indicate that the initial symptoms become first noticeable when the animals are driven for some distance. It is thought to be contagious and causes severe economic losses. An interesting feature of the disease consists of an extensive overgrowth of the pulmonary epithelium, but it is not known whether this proliferation constitutes the primary lesion nor has its tumor-like structure been studied. In view of the general similarity between this overgrowth and the proliferation of bile ducts in coccidiosis, Sir Arnold Theiler has suggested the possibility of the local action of some epithelial parasite susceptible of microscopic demonstration.

It was only upon my return from South Africa after comparing the lungs of jagziekte animals with the presumably normal ones of American sheep not affected by jagziekte, that evidence was found tending to show that the proliferations of epithelium are secondary to changes in the interalveolar tissue. It is proposed to describe briefly these primary changes and to give, in a second paper, a description of the origin and tumor-like character of the epithelial proliferations and of the subsequent changes.

* Fourth contribution by the South African Expedition of The Rockefeller Institute for Medical Research.

The observations were made in the laboratory of the Department of Agriculture at Onderstepoort and at New York. Cordial thanks are due to the Government of the Union of South Africa, to Sir Arnold Theiler, and to the members of his staff for the many courtesies extended.

Preliminary to this description a short summary of our knowledge of the disease, as contributed by Hutcheon,[1] Robertson,[2] and particularly by Mitchell,[3] may be helpful.

Etiology.—Hutcheon considered jagziekte to be infectious "under certain climatic conditions" and Robertson described peculiar crescent-shaped bodies with rounded ends in smears made from the lesions. These he thought to be of protozoan nature and suggested a resemblance to the crescents of subtertian malaria. But Mitchell, with much more extensive material at his disposal, failed to observe them and laid aside the parasitic theory, concluding from his experiments "that the conditions of transmission point to a specific virus." Experimental proof of the existence of a virus in animals suffering from the disease is lacking. It has never been possible even to transmit jagziekte to healthy sheep by the injection or inhalation of blood and diseased tissue.

Pathology.—According to Mitchell, jagziekte is essentially a chronic catarrhal pneumonia which progresses in the affected lung by continuity and contiguity. It is characterized by the presence of lymphoid nodules which show a marked tendency to infiltrate the surrounding tissue. These may develop in connection with the bronchi or in the interalveolar tissue. There is also bronchopneumonia with typical exudation of catarrhal products into the alveoli, interstitial fibroid changes, bronchitis and peribronchitis, and thickening of the arterial walls. As a result a general fibrosis of the affected portions ensues which renders the lesions hard and dense. Compensatory changes occur in the remaining tissue; *viz.*, emphysema, atelectasis, and pleuritis.

Mitchell found considerable variability in the occurrence of "proliferation of the bronchi." In his total of fifteen cases he described the proliferation as very marked in two, well marked in three, present in two, not marked in two, not apparent in one, absent in two, while in the remaining three he made no comment regarding its presence or absence. Moreover, in his general summary,[4] which has been abbreviated in the above paragraph, he did not apparently consider the proliferation of bronchi worthy of mention.

Epidemiology.—Jagziekte has been recognized as a specific and very destructive disease of sheep since about 1893, although it probably occurred earlier. It is prevalent in many districts of the Cape province, Orange Free State, and the Transvaal, but has not been reported in the more humid parts of Natal. There is evidence that the character of the grazing does not determine the incidence of the disease because it is found on grass, Karoo, bush veld, and mixed veld. The

[1] Hutcheon, D., Diseases of stock in South Africa, Pretoria.

[2] Robertson, W., *J. Comp. Path. and Therap.*, 1904, xvii, 221.

[3] Mitchell, D. T., *3rd and 4th Rep. Director Vet. Education and Research* (Sir Arnold Theiler), 1915, 585.

[4] Mitchell,[3] p. 600.

water supply of infected farms is equally variable, being obtained in some cases from above dams, in others from rivers or bore holes. Mitchell states that "the greatest mortality from the disease occurs in particularly wet years and [that] more cases develop in the flock during the rainy season than during the dry season." All breeds of sheep appear to be equally susceptible, males as well as females. The disease occurs most frequently in sheep about 3 years old. It has never been reported in lambs. Mitchell estimates the average mortality in the infected areas at 1.6 per cent per annum. This percentage does not vary greatly from year to year. Cases usually occur singly and no instance is on record in which large numbers of sheep in the same flock were affected simultaneously, although unusually severe outbreaks were reported in 1911–12.

The general belief that jagziekte is transmitted by contact is based upon field observation and direct experimentation. It is said that the disease spreads to clean areas by the importation of infected sheep and that kraals, in which sick animals have been kept, retain the infection and are able to transmit it to healthy sheep. The disease is known to be insidious and may be passed on to other sheep before any symptoms become apparent in the newcomers which carry it. It is this fact that makes laboratory experimentation so difficult and inconclusive because of the possibility that seemingly healthy animals have already contracted the disease before being placed in contact with sick sheep. The lesions appear from 3 to 5 days after contact (Mitchell). Under natural conditions death occurs in from 2 to 8 months.

Course.—There is a progressively increasing respiratory distress, followed by catarrhal discharge from the nose, cough, moist bronchial râles, loss of appetite and of weight, progressive muscular weakness, anemia, extreme emaciation, and death, with little or no fever at any time.

Differential Diagnosis.—The disease may be distinguished from the caseous lymphadenitis of Nocard by the above mentioned increased respiratory movements on exercise, cough, nasal discharge, and bronchial râles. Similar clinical manifestations may be produced by extensive infection of the lungs with the cysts of *Tænia echinococcus* but these may always be definitely identified post mortem.

Prognosis.—Death is apparently inevitable although life may be slightly prolonged by keeping the animals under good conditions and on a nutritious diet.

Prophylaxis.—The immediate slaughter of all cases is recommended.

Material and Methods.

Abundant material was placed at my disposal by Sir Arnold Theiler, and the members of his staff, which may be classified as follows:

1. Tissues from sixteen autopsies made on jagziekte sheep by the officers on duty.

2. Portions of the lungs from thirteen sheep, preserved in formalin, sent to the laboratory for examination, and diagnosed as jagziekte.

3. Parts of the lungs and bronchial lymph glands from thirty-eight sheep which died from bleeding in the preparation of blue-tongue vaccine.[5] These sheep came from a flock in which cases of jagziekte were occurring from time to time. Four of them exhibited early lesions, the existence of which was not suspected during the life of the animals.

4. For controls, the lungs of six sheep which suffered from heartwater,[6] and of three sheep which were carefully examined by Dr. Steck before death, and pronounced to be apparently normal.

5. As additional controls clearly free from even the earliest of jagziekte lesions, the lungs of thirty-eight sheep obtained in New York where they were sent from the Ozark region of the Middle West.

In the case of the first three autopsies, the heart, spleen, liver, kidneys, suprarenals, and gastrointestinal tract were examined in addition to the lungs merely to afford material for verification of the statements of Mitchell that the significant lesions of the disease are restricted to the lungs. Subsequently the lungs alone were studied, together with the bronchial lymph glands when it seemed desirable.

For routine purposes the tissues were fixed in Zenker's fluid or in 10 per cent formalin and were stained by Giemsa's method, or with hematoxylin and eosin. Special methods were used in the identification of cell types and in the search for microorganisms, including Levaditi's silver method for spirochetes. A total of 504 samples of tissue was thus examined in sections, to which may be added 13 blocks of tissue which were cut into serial sections occupying from 41 to 91 slides each. These latter were used in order to reconstruct roughly the topography of typical proliferations. Smears prepared in several ways were also studied and living tissues were examined in physiological saline solution by direct and oblique illumination.

The Primary Lesions.

The difficulty, or impossibility, of distinguishing clinically between animals in the early stages of jagziekte and other actually healthy animals offered an obstacle in the determination of the primary lesions.

At the time that tissues were being collected (July, 1924, to January, 1925) the disease was appearing with considerable frequency among the sheep kept at Onderstepoort. A thorough examination was accordingly made of the lungs and mediastinal lymph glands of thirty-eight sheep which before death did not exhibit noticeable symptoms of jagziekte, although some of them were not specially

[5] Blue-tongue is a kind of catarrhal fever of sheep peculiar to South Africa.

[6] Heartwater is a specific febrile disease which affects sheep, goats, and cattle in South Africa and is caused by *Rickettsia ruminantium* transmitted by the tick *Amblyomma hebræum* (Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 231, 253).

scrutinized for the disease. Four of these at autopsy showed distinct lesions characterized by variable degrees of epithelial proliferation and pneumonia, while others seemed to differ in less noticeable respects from the normal.

In order to ascertain the actual status of these suspected differences the lungs of an equal number of apparently normal sheep shipped to New York from the Ozark region of the Middle West (prairie country) were examined by the same methods. Very distinct differences were noted between the pulmonary tissue from these sheep, which were free from jagziekte, and that from the South African sheep, them-

TABLE I.

	American sheep.	South African sheep.
Thickening of interalveolar tissue.	Absent.	Present in 13.
Accumulation of macrophages and lymphocytes in interalveolar tissue.	"	" " 11.
Penetration of macrophages into alveolar lumen.	Present in 5.	" " 11.
Accumulation of polymorphonuclear leucocytes in interalveolar tissue.	Absent.	" " 4.
Penetration of polymorphonuclear leucocytes into alveolar lumen.	"	" " 4.
Peribronchial lymphocytic infiltration.	Present in 30.	" " 22.
Epithelial proliferation.	Absent.	" " 4.
Vascular congestion.	"	" " 11.
Occurrence of foreign material.	Present in 3.	" " 16.
" " bacteria.	Absent.	" " 6.

selves apparently free from the disease but coming from flocks in which the disease was certainly endemic. A glance at Table I shows that the latter exhibited a wider variety of lesions which were also of greater severity. That this circumstance is not due to the elimination of diseased animals by the inspector in New York may be accepted, because the tissues were removed before inspection took place. Both sets of animals were killed by bleeding.

The changes in the lungs of the South African sheep, which were never noticed in the American sheep and which may therefore be of significance in the etiology of jagziekte, center in the interalveolar tissue. This is noticeably thickened in thirteen out of thirty-eight

animals; that is to say, in about 33 per cent. The extent of these first alterations may be estimated by comparing Figs. 4 to 7 with Figs. 1 to 3, which latter represent approximately the normal appearance of the interalveolar tissue in American sheep.

The thickenings do not take place evenly throughout the lungs but are first seen in scattered foci 1 to 15 mm. in diameter. These may be directly beneath the pleura or may occur more deeply in the pulmonary tissue, but no definite relationship to the bronchioles or to the interlobular septa was observed.

They are caused by the accumulations of large phagocytic ameboid cells possessed of abundant cytoplasm and simple spherical nuclei which have been variously called macrophages (Metchnikoff), polyblasts (Maximow), endothelial leucocytes (Mallory), and mononuclear leucocytes (Ehrlich). They are accompanied by many lymphocytes of variable size, the larger ones of which resemble them closely, and by a few polymorphonuclear leucocytes. None of these cells exhibit signs of mitotic division.

No evidence was found that the macrophages are produced through the multiplication of the endothelial cells of the alveolar capillaries as described by Permar.[7] Probably they are chiefly of hematogenous origin. In some instances they were seen within the capillary lumina in contact with erythrocytes and free from the lining endothelium (Fig. 9). In all likelihood they may also arise from cells resident in the interalveolar connective tissue, in other words from the histiocytes of Kiyono, or the rhagiocrine cells of Renaut (to employ only a few of the available synonyms). That they may be derived from the lymphocytes which are likewise present in the interalveolar tissue is to be considered as a third possibility in view of Maximow's[8] observation that freshly emigrated blood lymphocytes are capable of transformation into large phagocytic ameboid cells; that is to say, in this case, into macrophages. The lymphocytes, in turn, like most of the macrophages themselves, probably emigrate from the alvolear capillaries, since, although we have no specific information regarding the pulmonary lymphatics of sheep, Miller[9] has been unable to find in

[7] Permar, H. H., *J. Med. Research*, 1920–21, xlii, 147, Plate IV.
[8] Maximow, A. A., *Physiol. Rev.*, 1924, iv, 533.
[9] Miller, W. S., *Am. Rev. Tuberc.*, 1919–20, iii, 193.

man any lymphatics in the walls of the air sacs beyond the ductuli alveolares. Proximal to this point there are no unusual accumulations of lymphocytes in these early stages in the lesions of the South African sheep. Indeed the American sheep show much more extensive peribronchiolar lymphocytic infiltrations than they do. A comparison of the walls of the bronchioles and of the bronchi of the two sets of animals did not reveal any instructive differences between them which might suggest the spread of infection by the lymphatic route.[10, 11]

Coincident or antecedent to this infiltration of the interalveolar tissue a variable degree of similarly localized dilatation of the alveolar capillaries is to be noted (see Figs. 10, 12, and 13) which conforms with Mitchell's statement that the primary lesions of jagziekte are hyperemic. At the time when this dilatation is present the macrophages commence to migrate into the alveolar lumina in numbers which exceed the normal as seen in American sheep. The exudate at this time contains very few polymorphonuclear leucocytes. One macrophage in the act of entry into an alveolus is represented in Fig. 10. Its typically rounded nucleus has assumed a kind of hour-glass glass shape, half inside and half outside of the alveolus, and has been preserved in this state by the fixative.

In this new location the macrophages accumulate in large numbers where they have been frequently reported in diseases of the lungs. They are the cells so often referred to as mononuclear phagocytes of the lungs and as epithelioid cells.[12] They may be distinguished from desquammated epithelial cells, with which some investigators have identified them,[13–15] by their characteristic structure. Their nuclei are now less spherical and more kidney-shaped. By staining with iron-hematoxylin a centrosome, or a diplosome, is revealed on the side of the nuclear concavity. These cell organs are always centrally

[10] Winternitz, M. C., Smith, G. H., and Robinson, E. S., *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 63.

[11] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 445.

[12] I am indebted to Dr. H. S. Willis for the details of a special method for identification of epithelioid cells.

[13] Briscoe, J. C., *J. Path. and Bact.*, 1908, xii, 66.

[14] Sewell, W. T., *J. Path. and Bact.*, 1918–19, xxii, 40.

[15] M'Fadyean, J., *J. Comp. Path. and Therap.*, 1920, xxxiii, 1.

placed and are surrounded by a radial arrangement of cytoplasmic granules. Coloration with fuchsin and methyl green after fixation in Regaud's fluid brings to light numerous rod-like mitochondria in the peripheral cytoplasm in company with droplets of neutral fat which may be stained in frozen sections with Scharlach R. These epithelial cells, which may still with propriety be termed macrophages, differ only from the typical macrophages, or large mononuclears (monocytes) from which they arise, by changes incident to the amount, character, and condition of the materials which they have ingested, as has been clearly shown in the case of the epithelioid cells of tuberculous lesions by Lewis, Willis, and Lewis.[16] And furthermore, like those in tuberculous lesions, they also give rise to multinucleate giant cells (Fig. 14) which often attain considerable dimensions, particularly in the later stages of jagziekte.

It is in hyperemic areas of this kind with interalveolar infiltrations of macrophages, some of which penetrate into the alveolar lumina that the earliest epithelial proliferations, which are so characteristic of jagziekte, commence (Fig. 8). That these infiltrative and exudative changes constitute in reality the primary lesions of the disease may be accepted from the following observations:

1. That they were found in thirteen out of thirty-eight sheep which before death (in the preparation of blue-tongue vaccine) failed to exhibit noticeable symptoms of jagziekte although the disease was appearing from time to time in sheep belonging to the same flock; whereas they were absent in the lungs of an equal number of American sheep not subject to jagziekte.

2. That in all of the thirty-three cases of pronounced jagziekte examined in which the disease was readily detected clinically, the epithelial proliferations without exception arose in areas of the lungs thus modified.

3. That no epithelial lesions were found, although exhaustive search was made, in the lungs of thirty-four South African sheep which did not contain these preliminary infiltrative and exudative alterations.

An interesting deduction follows; namely, that since these primary

[16] Lewis, M. R., Willis, H. S., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1925, xxxvi, 175.

lesions occur in the absence of typical signs of jagziekte it is possible that some, perhaps many, animals in the early and thus unrecognizable stages of the disease do recover, although after the development of positive symptoms a fatal termination may be inevitable, as is generally supposed.

On a purely morphological basis no satisfactory explanation could be found for the prevalence of these primary lesions in the lungs of sheep of a flock subject to jagziekte, but brief reference may be made to the incidence of foreign material and of bacteria detectable microscopically.

The foreign material was usually observed within the peribronchial lymphatics and less frequently within macrophages in the thickened interalveolar tissue (Fig. 11). It was identified in the lungs of sixteen sheep and in the mediastinal lymph glands of eleven others, from which we may infer its presence in a high percentage of the flock. It was found to occur both during the humid rainy weather of midsummer (January) and also during the driest and most dusty period of winter (August), as well as in sheep from different localities.

The material differs from that noted in the lungs of three of the thirty-eight American sheep. It is readily identified in sections stained by Giemsa's method in which it assumes an intense, rosy red color. It remains unmodified in color after treatment with hematoxylin and eosin. Its properties were studied in unstained sections by methods advised by Mavrogordato[17] on the basis of his experiments upon the effect of foreign materials upon the lungs. It was found to occur in the form of biconvex lens-shaped bodies with sharp edges from 3 to 15 microns in diameter and 1 to 5 microns in thickness. These do not offer sufficient resistance to the knife to cause tears in the sections. When the bodies are broken, as was often the case, the lines of fracture are straight and not rounded. No definite crystalline facets were noted. The bodies are translucent and faintly yellow in color. They are not dissolved by the action for 1 hour at 60–65°C. of the following reagents: (1) acetone, (2) 95 per cent alcohol, (3) 95 per cent alcohol plus 1 per cent of hydrochloric acid, (4) half absolute alcohol and chloroform, and (5) 0.5 per cent aqueous hydrochloric acid. Evidently the bodies do not consist of cholesterol or

[17] Mavrogordato, A., *J. Hyg.*, 1918, xvii, 439.

some other material likely to be produced locally through pathologic changes. On the contrary, their properties and the fact that they are actively engulfed by macrophages indicate clearly that they are of exogenous origin.

In addition to this substance, irregular fragments of vegetable tissue were sometimes observed, which were likewise absent from the lungs of American sheep; but their occurrence was not sufficiently constant to indicate the advisability of further study. They are mentioned only because it is important to note that the lungs of South African sheep are subject to the inhalation of an unusual variety of substances which may have an irritating action.

Though it is known that the lungs normally possess a varied bacterial flora[18,19] the observation is interesting that the lungs of six of the South African sheep contained bacteria demonstrable histologically, whereas none were seen in the lungs of American sheep not subject to the disease. At least two kinds were noted: a Gram-negative bacillus, about 5 by 1 microns, which was colored faintly by Giemsa's method, and a Gram-positive diplococcus, about 1 by 2 microns, which was stained deep blue by Giemsa's technique. Both were often associated with the above mentioned foreign material and perhaps gained entrance with it.

SUMMARY.

The lungs of South African sheep, in a district in which jagziekte is appearing sporadically, differ from those of normal American sheep. Their structure is in general variable. They are more subject to the action of bacteria, demonstrable histologically, and to the effect of inhaled foreign material. The most significant difference, however, centers in the interalveolar tissue, which, in about 33 per cent of the animals, is definitely thickened beyond the range of variation in this direction observed in American sheep. The thickenings occur in localized areas several millimeters in diameter. They begin with engorgement of the alveolar capillaries and accumulation of macrophages and of lymphocytes. Many of the macrophages pass into the

[18] Jones, F. S., *J. Exp. Med.*, 1922, xxxvi, 317.
[19] Spray, R. S., *J. Infect. Dis.*, 1922, xxxi, 10.

alveolar lumina and assume the appearance of typical epithelioid cells. These infiltrative and exudative changes are primary to the epithelial proliferations which always arise in tissues modified in this way and which it is proposed to describe in a second paper.[20]

EXPLANATION OF PLATES.

PLATE 14.

FIGS. 1 to 3. The normal range of development of the interalveolar tissue as seen in American sheep not subject to jagziekte. × 120.

FIG. 4. Beginning infiltration of interalveolar tissue in a South African sheep. × 120.

PLATE 15.

FIGS. 5 to 7. Progressive stages in the infiltration of the interalveolar tissue with macrophages and lymphocytes. × 120.

FIG. 8. Early alveolar proliferations in an area thus modified. × 120.

PLATE 16.

FIG. 9. A macrophage within an alveolar capillary. × 1,000.

FIG. 10. Another macrophage in the act of emigration is seen in the center. × 1,000.

FIG. 11. Foreign material undergoing phagocytosis by macrophages in the thickened interalveolar tissue. × 1,000.

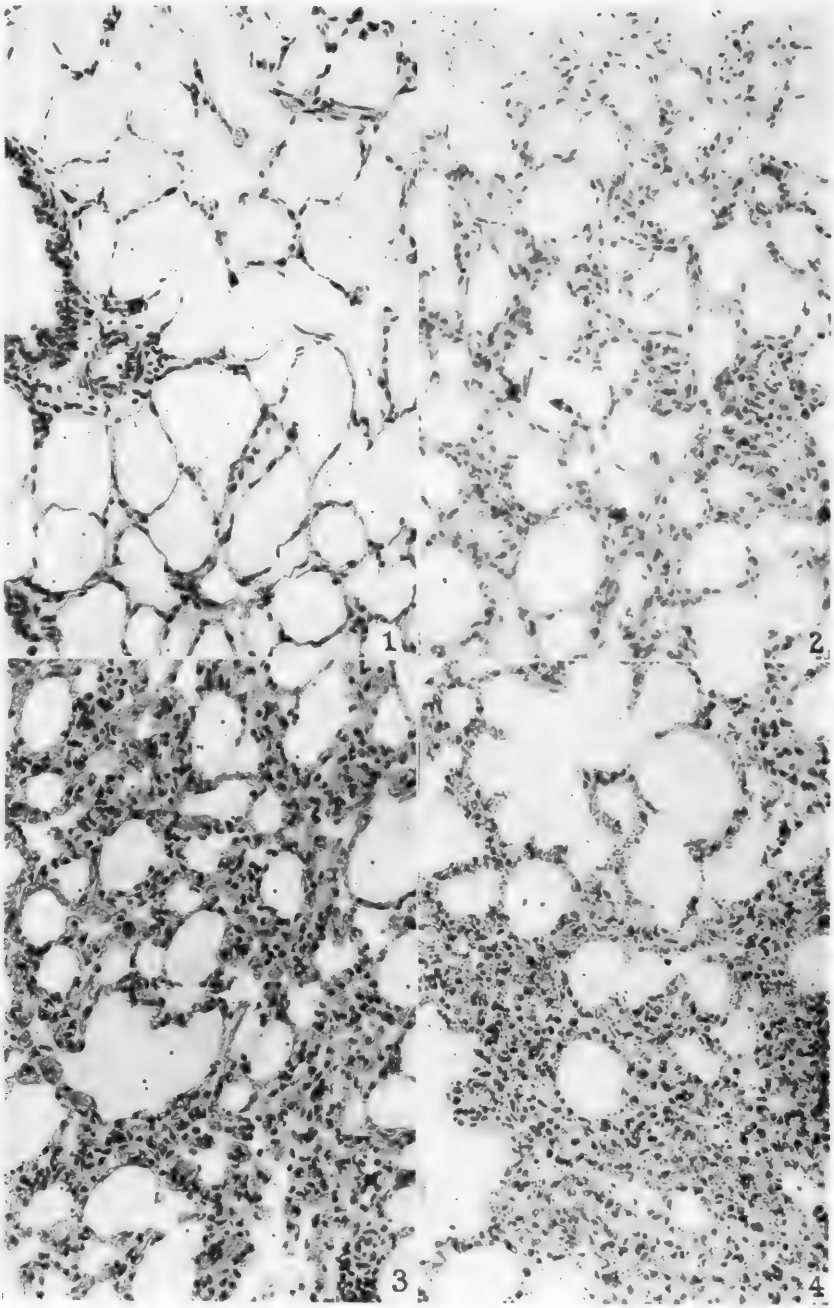
FIG. 12. Macrophages and lymphocytes of variable size in the interalveolar tissue. × 1,200.

FIG. 13. Macrophages (epithelioid cells) within the lumen of an alveolus. Note the congestion of the alveolar capillaries. × 1,200.

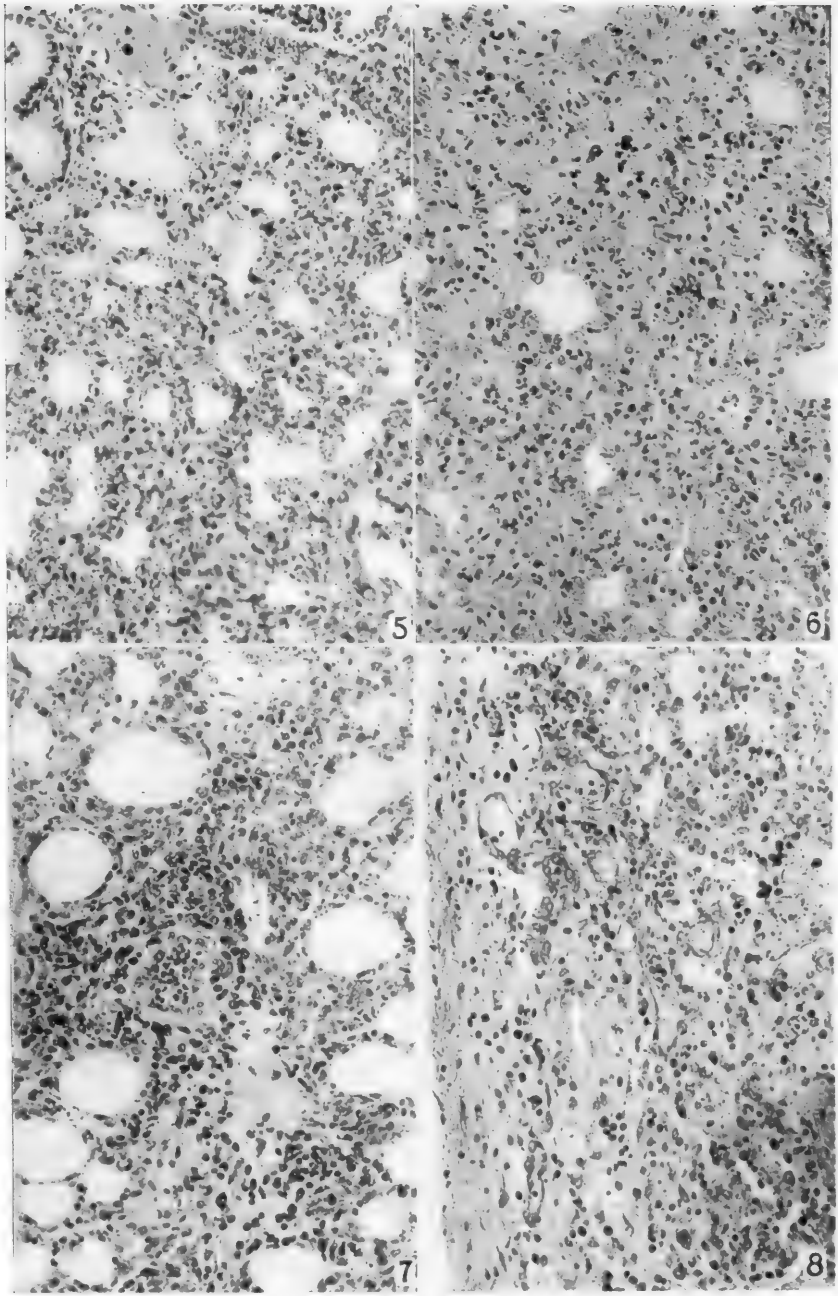
FIG. 14. A small multinucleated phagocytic group cell within the lumen of an alveolus. × 1,200.

---

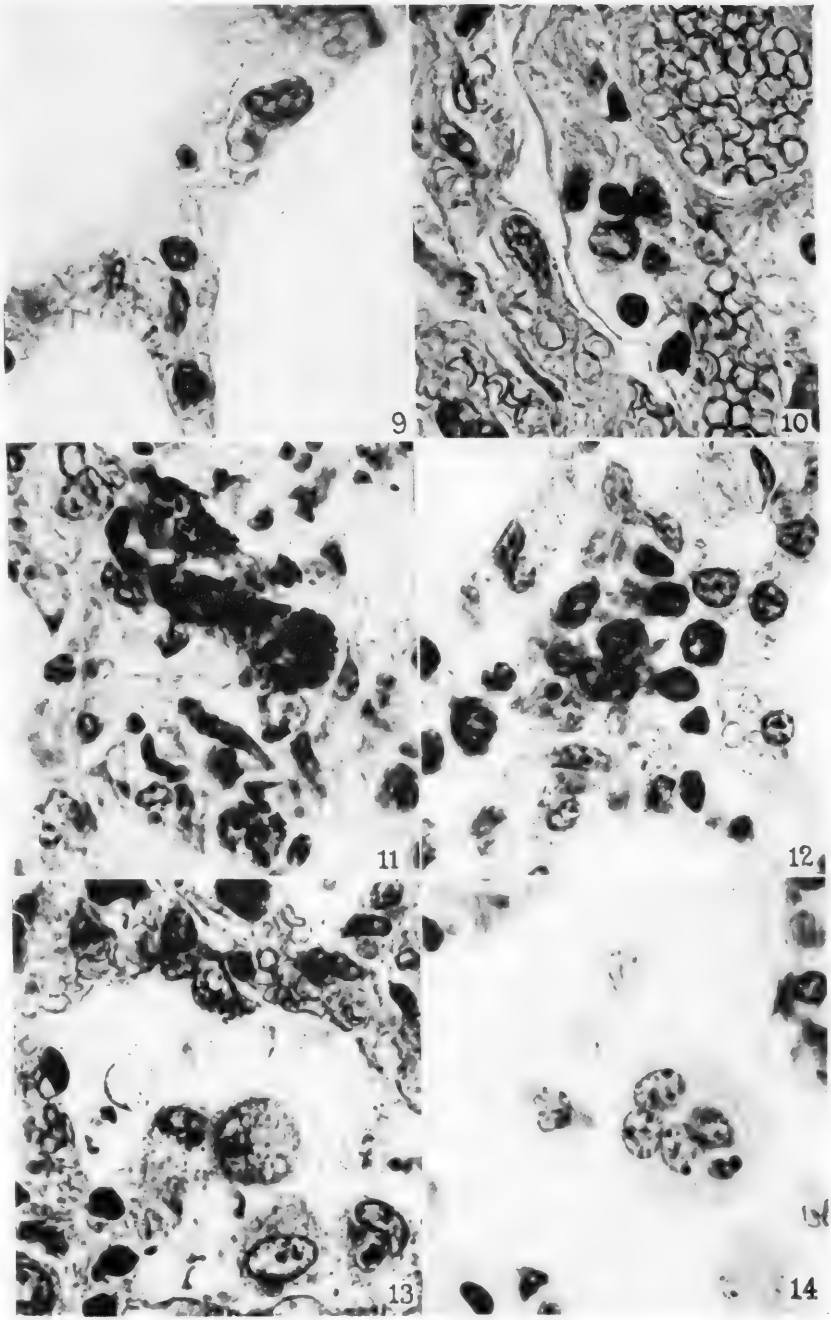
[20] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 335.



(Cowdry: Etiology of jagziekte. I.)



(Cowdry: Etiology of jagziekte. I.)



(Cowdry: Etiology of jagziekte. I.)

STUDIES ON THE ETIOLOGY OF JAGZIEKTE.

II. Origin of the Epithelial Proliferations, and the Subsequent Changes.*

By E. V. COWDRY.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 17 to 19.

(Received for publication, May 25, 1925.)

In my first paper on jagziekte the primary lesions in apparently normal sheep from flocks in which the disease is endemic were described.[1] They develop through a local congestion of the alveolar capillaries, accompanied by the accumulation of macrophages and of lymphocytes in the interalveolar tissue, and the penetration of the former in large numbers into the alveolar lumina. It is only in portions of the lungs modified in this way that the epithelial proliferations, which are so characteristic of jagziekte, arise. The proliferations develop in scattered island-like masses, as illustrated in Fig. 1. They soon become confluent, and large areas of pulmonary tissue assume an adenomatous appearance (Fig. 2). In certain cases, on the other hand, growth takes place in the form of densely packed masses of cells (Fig. 3). Fibrosis occurs in the more central parts of the lesions, and the animals usually die from bronchopneumonia.

Origin of the Epithelial Proliferations.

Coincident with the commencement of epithelial growth there is a reduction in the alveolar blood supply which later becomes extreme.

* Fifth contribution by the South African Expedition of The Rockefeller Institute for Medical Research.

The observations were made in the laboratory of the Department of Agriculture at Onderstepoort and at New York. Cordial thanks are due to the Government of the Union of South Africa, to Sir Arnold Theiler, and to the members of his staff for the many courtesies extended.

[1] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 323.

This may be roughly estimated by comparing Figs. 5 and 8, in which the capillaries are practically invisible, with Figs. 10, 12, and 13 of the first paper which illustrate considerable engorgement.

The first indications of unregulated growth consist of the enlargement and multiplication of cells bordering a portion of the lumen of an alveolus. The cells may either assume a papillomatous arrangement (Fig. 6), or may remain attached to the alveolar wall over a larger area (Fig. 8). That they are actually of alveolar origin and are not merely enlargements of the usual cubical cells situated at the point of termination of a bronchiole was proved in several instances by following such small islands of proliferating tissue through in serial sections which revealed the fact that they are in continuity on all sides with flattened alveolar epithelium, in which nuclei are very difficult to detect.

It may be said that these cells are not only atypical in their shape, but also in their internal architecture, although the original conditions are not easily established for comparison, because there is so little agreement regarding their normal structure. Ogawa[2] is of the opinion that two distinct types exist, a small nucleated cell and a larger plaque-like non-nucleated structure. We have no information regarding the mitochondria of such elements in the fully developed state; but it is known that the cubical alveolar cells of the fetal lung are provided with rod-like and filamentous mitochondria which are distributed evenly throughout their cytoplasm.[3,4] In the proliferating cells under discussion the mitochondria have considerably enlarged in the form of droplets and short rods which have become heaped up in the distal cytoplasm.

The second change is well illustrated in Fig. 8, and from an examination of many specimens seems to take place abruptly. It will be seen that the proliferating cells to the right, in the figure, have doubled both the size of their cytoplasm and that of their nuclei as compared with contiguous cells to the left. Their outlines bulge and their chromatin is deeply stained. They resemble the adenoma-like cells characteristic of the older lesions of jagziekte.

[2] Ogawa, C., *Am. J. Anat.*, 1920, xxvii, 315.
[3] Stewart, F. W., *Anat. Rec.*, 1923, xxv, 181.
[4] Fauré-Fremiet, E., and Dragoiu, J., *Arch. anat. micr.*, 1923, xix, 411.

While the hypertrophied cells remain in a single layer, which has not as yet completely encircled the lumen of the alveoli, a definite immigration of polymorphonuclear leucocytes sets in. The nuclei of these leucocytes may be distinguished at the base of the growth in Fig. 6, and at the center of Fig. 7. They occasionally penetrate into the cytoplasm of the epithelial cells. Many of them are phagocytized by the macrophages, as illustrated in Fig. 5. During the metamorphosis of the alveolar epithelium into a single continuous layer of cubical, or more frequently of columnar cells, the macrophages and lymphocytes persist in the interalveolar tissue while the cellular exudate is reduced in amount. The local disappearance of the cellular exudate is illustrated under lower magnification in Fig. 2. Indeed, the contrast between these clear lumina and the surrounding alveoli containing many phagocytic macrophages is often quite striking.

The reduction in the cellular exudate is probably linked up with the diminution in blood supply, already mentioned, and with the change in character of the lining alveolar epithelium. That this now forms a continuous and much folded investment of cubical or columnar cells is strong evidence either that the whole surface of lining epithelium is capable of proliferation, or that regeneration takes place through lateral extension from rather isolated clumps of small nucleated cells as claimed by Ogawa.[2] At this stage the proliferations resemble closely those described by Sir John M'Fadyean in verminous pneumonia of sheep.[5] Indeed, a specimen kindly sent to me by Sir John would be difficult to distinguish from jagziekte except by a consideration of its history.

The formation of several layers of cells, which now become quite atypical in structure, begins to take place in the more central parts of the altered tissue. Parallel with it the infiltration of the interalveolar tissue, which constitutes the first sign of the disease,[1] continues without abatement, and is supplemented by a proliferation of fibroblasts (although few mitoses are observed), resulting in the development of a kind of stroma (Fig. 4) and in more or less extensive interalveolar fibrosis, which interferes further with the blood supply, and which will be mentioned again later. There are also developed,

[5] M'Fadyean, J., *J. Comp. Path. and Therap.*, 1894, vii, 31; 1920, xxxiii, 1.

in rare instances, in the same interalveolar location certain foci, 2 to 10 mm. in diameter, of peculiar myxomatous tissue, the existence of which was first pointed out to me by Sir John M'Fadyean, who courteously examined my preparations.

The newly formed epithelial cells often give rise to irregular masses of tissue jutting out into the alveolar lumina, as represented in Fig. 10. This is similar to the epithelial proliferation following influenzal pneumonia, described by Winternitz, Wason, and McNamara,[6] and illustrated in their Fig. XLIX. But in influenzal pneumonia the proliferations are more neoplastic in their invasive properties, though much less extensive in growth. The cells in jagziekte may also change their character within a single alveolus from a large columnar type to a smaller flattened, stratified type (see Fig. 9). Or they may assume the form of dense sheet-like masses, as represented under lower magnification in Fig. 3. The edges of such masses often push their way through the wall of an alveolus into the surrounding connective tissue (Fig. 11).

In this description stress is placed upon the alveolar origin of the proliferations, which obtains throughout the altered tissue, although it should be noted that the bronchiolar epithelium also contributes to the adenoma-like formations.

In the case of the bronchiolar epithelium a much smaller surface is involved and the proliferation is, as far as it has been possible to ascertain, only secondary, occurring after the alveolar proliferation is well under way. Perhaps the commonest mode of growth is by the development of extensive folds in the walls of the bronchioles. At the beginning of the process mucigenous cells may be seen, but later they disappear. Or, on the other hand, the growing cells may form a plaque-like mass attached to the inner wall of a bronchiole, which, by gradual enlargement, sometimes entirely occludes its lumen by the formation of a dense plug of cells without any semblance of orderly arrangement. Overgrowths in this location are sometimes of unusually atypical structure (Fig. 12).

Proliferation of the epithelium of the bronchi is much less frequent. This, perhaps, finds explanation in the fact that they are still further

[6] Winternitz, M. C., Wason, I. M., and McNamara, F. P., The pathology of influenza, New Haven, 1920.

removed from the interalveolar tissue in which the primary changes occur. Desquamation of the bronchial epithelium is fairly common; whereas this process is rarely met with in the alveoli, which are often filled with cells of hematogenous origin, as mentioned in the first paper on jagziekte.

Concerning the epithelial proliferations in general, two further points remain to be mentioned. First, the remarkable infrequency of mitoses in a growth which has every appearance of being so vigorous and luxuriant; second, the absence of spread by metastasis to other parts of the body, even to the neighboring lymphatic glands.

The Subsequent Changes.

The proliferating epithelium becomes so extensive that areas several inches in diameter would be difficult to identify as altered pulmonary tissue were it not for their location. They resemble, if only superficially, certain mammary gland adenomata (Fig. 2). Extensive foci of lymphocytic infiltration, chiefly peribronchiolar in location, upon which Mitchell[7] lays stress in his account of the pathogenesis of the disease, may or may not occur in my preparations. When present they rarely exceed in extent the normal, as seen in American sheep, although their centers have a greater tendency to undergo necrosis. The fibrosis continues and results in widespread consolidation. The area of normal respiratory epithelium is thus greatly reduced. Death usually occurs from bronchial pneumonia, which may conceivably be an expression or continuation of the primary infiltrative and exudative changes, which latter are in the course of the disease to some extent overshadowed by the superimposed epithelial proliferations.

Coexistence of Lesions of Different Kinds.

In the foregoing pages an attempt has been made to describe the sequence of changes in jagziekte, but it is not to be supposed that at any moment all parts of the lungs are similarly involved. On the contrary, after the appearance in multiple foci of the primary infiltrative and exudative changes, the process spreads into more normal

parts of the lungs, as Mitchell[7] has shown. In fairly advanced cases it is possible to find epithelial overgrowths, infiltration and proliferation of the interstitial tissues, and acute pneumonia, with exudation of polymorphonuclear leucocytes almost side by side.

Search for Microorganisms.

In view of the similarity, already referred to, between the epithelial proliferations in jagziekte and others described by M'Fadyean,[5] and also by Hofman[8] in verminous pneumonia of sheep, a thorough search was made for nematodes but without success, although many samples of tissue were examined from thirty-three sheep possessed of definite jagziekte lesions. Neither were any signs of nematodes detected in sheep exhibiting only the primary lesions, and in others belonging to the same flock. Mitchell's[7] observations also furnish evidence, of a negative character, that nematodes are not concerned in the production of jagziekte.

Except for the existence of the primary lesions above mentioned and the atypical character of the epithelial proliferations, there is also a superficial resemblance between the epithelial growths and the hyperplasia of hepatic bile ducts in coccidiosis. Eber[9] has, moreover, described a multiple adenoma of the sheep's lung which he suggests may be due to a protozoan-like organism. Although this adenoma resembles jagziekte only remotely,[10] the possibility was entertained that jagziekte itself might be caused by some kind of epithelial parasite acting locally, and, further, that the parasite might disappear from the tissues soon after the creation of a stimulus to abnormal growth or the suppression of some mechanism of regulation, and thus be very difficult to detect. But so many of the very earliest lesions were examined completely in serial sections that this hypothesis seemed improbable, at least in respect to the action of a parasite

[7] Mitchell, D. T., *3rd and 4th Rep. Director Vet. Education and Research* (Sir Arnold Theiler), 1915, 585.

[8] Hofman, V., *Časop. lék. česk.*,1923, lxii, 65; abstracted in *J. Am. Med. Assn.*, 1923, lxxx, 1279.

[9] Eber, A., *Z. Tiermed.*, 1899, iii, 161. (Due to protozoa? Suggested only.)

[10] The annular bronchial polyadenoma of unknown causation described by Ball (Ball, *J. méd. vét. et zootechn.*, 1907, lviii, 71) differs still further from jagziekte.

demonstrable microscopically. Neither was it possible to find any evidence of the action of a malaria-like microorganism, as suggested by Robertson.[11]

Taking also into consideration what little is known of the etiology of jagziekte, it was thought that the disease might conceivably be caused by spirochetes, the presence of which might not be revealed by ordinary methods of technique. Accordingly, many preparations were made by silver impregnation, as advised by Levaditi, both the concentration of the ingredients and the duration of the various steps being varied within wide limits, but without success. This was supplemented by prolonged studies of teased living tissues by dark-field illumination, and by methods designed for the demonstration of spirochetes in smears and in artificial culture media, likewise with wholly negative results.

Several kinds of bacteria were found in outspoken cases of jagziekte in addition to those mentioned in the lungs of a flock of sheep in which the disease was appearing sporadically.[1] They usually occurred free or within macrophages in the lumina of the alveoli and bronchi, and were often associated with extensive leucocytic infiltrations, sometimes with slight necrosis; but they were never found within the proliferating epithelium. In others words, the epithelial proliferations themselves are probably as free from directly causal microorganisms demonstrable microscopically, as are most hyperplasias and tumors.

DISCUSSION.

As a result of the foregoing observations several tentative suggestions regarding the etiology of jagziekte may be formulated. But before attempting to do so, brief reference may be made to the prevailing view that the disease is due to a specific virus, and that it is directly contagious.

This conception is based upon the experience of all trained observers who have studied jagziekte under natural conditions. It is known that it spreads to previously clean areas after the importation of infected sheep, and that kraals, in which sick animals have been kept, retain the infection and are capable of transmitting it to healthy

[11] Robertson, W., *J. Comp. Path. and Therap.*, 1904, xvii, 221.

sheep. It is also based upon the outcome of carefully planned contact experiments. But, like many diseased conditions of the internal organs which cannot be directly observed *in vivo*, there is evidence that animals may possess typical jagziekte lesions without the exhibition of noticeable clinical symptoms. Obviously the value of the experiments is lessened by this knowledge that the apparently healthy animals to which an attempt is being made to transmit the disease may already have contracted it. The logical method of approach to a study of its mode of transmission would be, not to bring animals from a district in which jagziekte has never been reported into one in which it occurs, for experimentation, as was done by Mitchell, but rather to bring sick animals into a region naturally free from the disease. But the practical obstacles to such a procedure are great.

It is conceivable that the consistent failure of investigators to transmit jagziekte by the direct inoculation or inhalation of diseased tissue into presumably healthy animals may indicate the operation, in the naturally occurring disease, of some predisposing factor or factors. Some color is lent to this hypothesis by the observation that the lungs of a flock of sheep in which jagziekte was endemic differed noticeably from those of an equal number of sheep from a region in which the disease did not exist.[1] The differences would seem to be significant. They center in the interalveolar tissue which, in about 33 per cent of the animals, is definitely thickened beyond the range of variation in this direction noted in American sheep. The thickenings occur in localized areas several millimeters in diameter. They are caused by the engorgement of the alveolar capillaries and by the accumulation of macrophages and of lymphocytes. Many of the macrophages pass into the alveolar lumina, where they assume the appearance of typical epithelioid cells. It was found that these infiltrative and exudative changes are primary to the proliferations of epithelium which always arise in parts of the lungs thus modified.

Primary lesions of this kind might well result from several stimuli of different types, spread over an appreciable interval of time. It is interesting to note in this connection that the South African sheep subject to jagziekte are also peculiar in the fact that foreign material was of much more frequent occurrence in their lungs than in those of American sheep. Judging only from a microscopic study, it may be

said that they are also more liable to bacterial invasion. It was further observed that the bacteria often gain entrance in company with the foreign material.

Since it was found that the infiltrative and exudative changes do not cease with the formation of the epithelial proliferations, which become so extensive as to lend a very remarkable appearance to the lungs, but rather progress and persist, side by side, with them, it is possible that the adenomatous condition is merely a kind of epiphenomenon. There is ample precedent for such an interpretation; for example, the hyperplasias of pulmonary epithelium which follow the ingestion of plant toxins,[12] bacterial infections,[6] nematode infections,[5,8] and diseases, like measles, of unknown causation.[13] Indeed, the observations of M'Fadyean, Hofman, and Eber, and of investigators dealing with jagziekte[14] may indicate that the pulmonary epithelium of sheep is peculiarly unstable, in other words, that it is unusually susceptible to growth stimuli or to influences tending to invalidate normal inhibitory mechanisms.

Furthermore, the coexistence in one and the same lung of many stages of the reaction and of areas of acute pneumonia, characterized by the accumulation and exudation of polymorphonuclear leucocytes points to the possibility that the lesions of jagziekte may result from a complex of influences acting together or successively over a considerable period of time. Although the epithelial proliferations may thus be supplementary, they contribute definitely to the death of the animals by the diminution of their respiratory area and of their vitality, thus rendering them still more susceptible to bacterial action.

While the exciting cause or causes of jagziekte remain so obscure, the suggestion is advanced that the lungs of a flock of sheep in which cases of jagziekte are frequently occurring, and which therefore include instances of the earliest lesions not susceptible of clinical diagnosis, should be systematically studied by cultural methods.

[12] Theiler, Sir Arnold, *7th and 8th Rep. Director Vet. Education and Research*, 1918, vi, 57.

[13] Hart, C., *Deutsch. Arch. klin. Med.*, 1904, lxxix, 108 (measles).

[14] See summary by du Toit, J. P., Die Jagziekte der Schafe, in Knuth and du Toit, J. P., Tropen-Krankheiten der Haustiere, Leipsic, 1921.

SUMMARY.

The epithelial proliferations, which are so typical of jagziekte, are chiefly of alveolar origin. They appear in areas of infiltration and exudation identical histologically with those occurring in sheep which are to all appearances normal but which are derived from flocks in which the disease is endemic.[1] They become very extensive and quite atypical in structure, but they never give rise to metastases, even to the adjacent lymph glands. The primary infiltration of the interalveolar tissue continues and may lead to fibrosis and extensive consolidation, or leucocytes may accumulate in large numbers, penetrate into the alveoli, and form areas of acute pneumonia. In fairly advanced cases of jagziekte it is common to find many stages of the reaction in one and the same lung. While it is possible that the disease may be due to a single and specific virus, and the epithelial lesions are certainly of a very special character, the pathological complex as a whole is such as might be caused by a variety of predisposing and exciting factors operating over a considerable period of time.

EXPLANATION OF PLATES.

PLATE 17.

FIG. 1. Epithelial proliferations arising in island-like masses in a region of the lung in which there has been an infiltration of macrophages and lymphocytes into the interalveolar tissue and some penetration of the former into the alveolar lumina. × 75.

FIG. 2. Growth of epithelium in the form of a single layer completely investing the alveolar lumina. × 75.

FIG. 3. Growth of epithelium as a dense mass many cells in thickness. × 240.

FIG. 4. Growth of epithelium and of stroma. × 240.

PLATE 18.

FIG. 5. Cellular exudate consisting of phagocytic mocrophages (epithelioid cells). × 1,000.

FIG. 6. Papillomatous epithelial growth arising from a small portion of the wall of an alveolus. × 1,000.

FIG. 7. A similar growth cut transversely with contained leucocytes. × 1,000.

FIG. 8. A small alveolar overgrowth in which the cells to the right have become greatly hypertrophied. × 1,000.

FIG. 9. A proliferation of alveolar epithelium in which the character of the

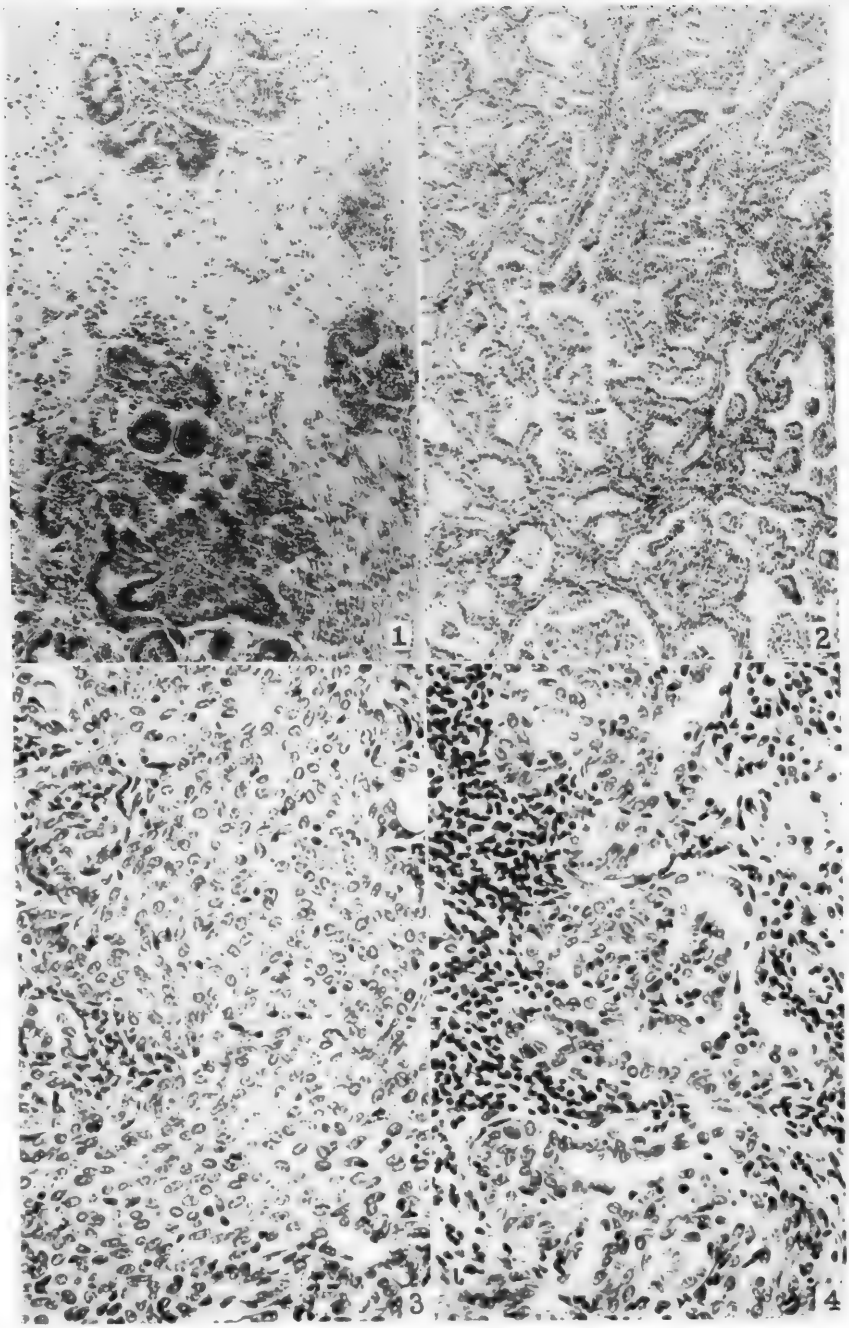
constituent cells changes from large columnar (below) to small and stratified (above). × 1,000.

FIG. 10. Irregular arrangement of growing cells with but little cytoplasm. × 1,000.

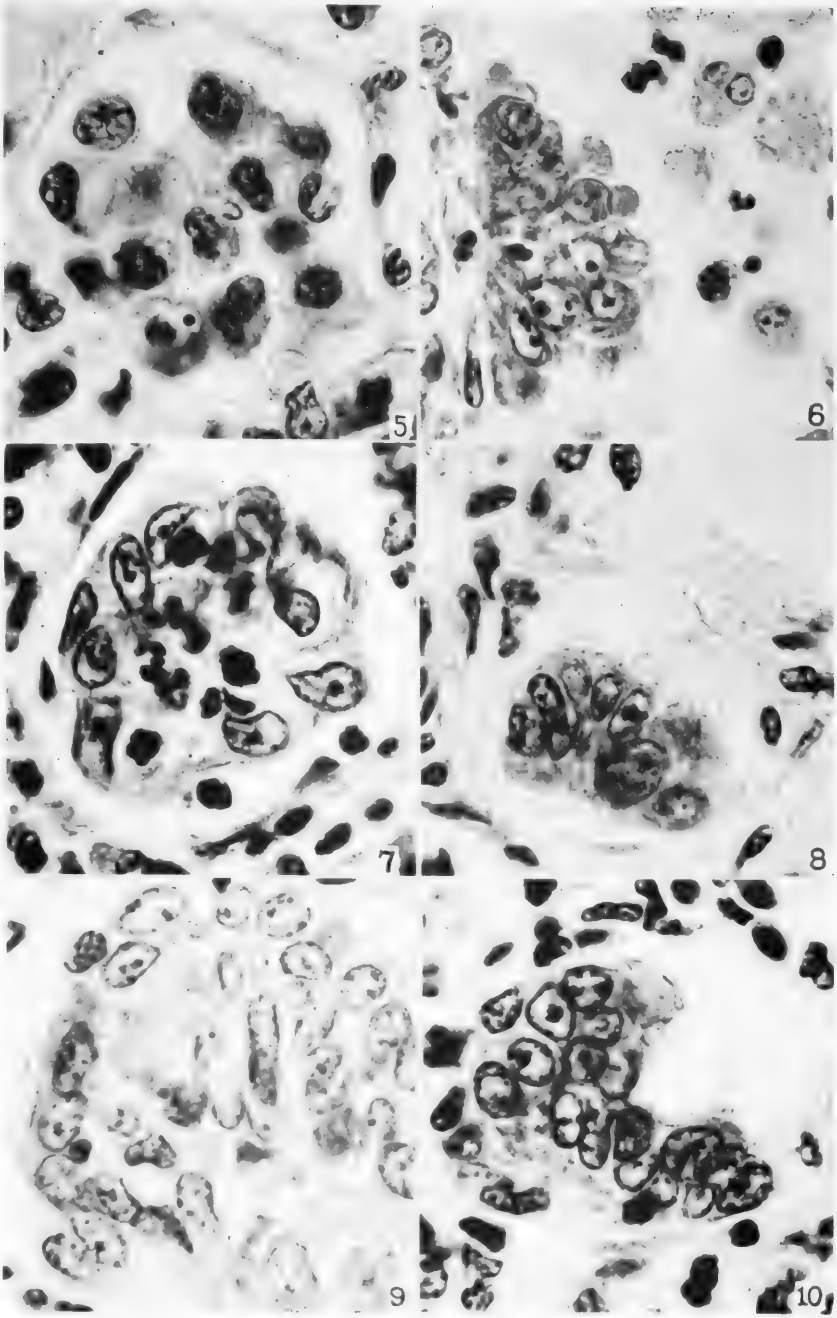
PLATE 19.

FIG. 11. A mass of epithelial tissue penetrating through the wall of an alveolus into the surrounding interalveolar tissue. × 1,000.

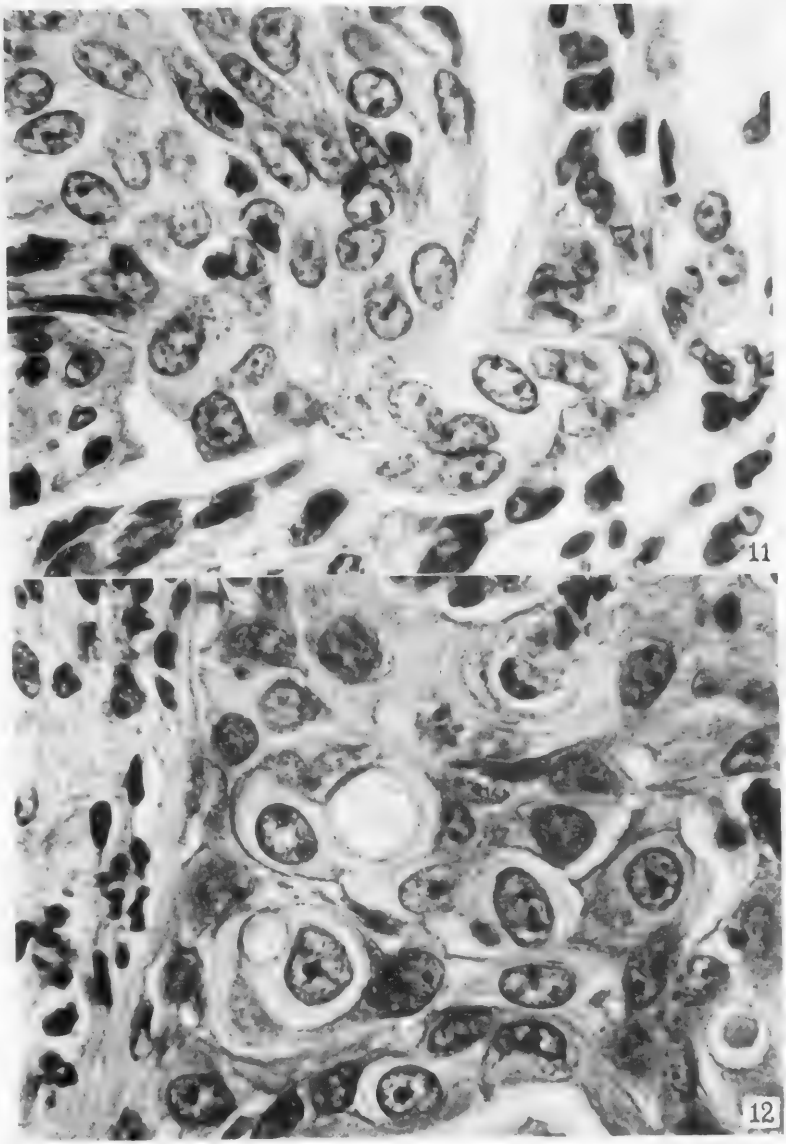
FIG. 12. Atypical growth of epithelial cells lining a bronchiole as seen in transverse section. × 1,000.



(Cowdry: Etiology of jagziekte. II.)



(Cowdry: Etiology of jagziekte. II.)



(Cowdry: Etiology of jagziekte. II.)

IMMUNOLOGICAL REACTIONS OF THE ISOLATED CARBOHYDRATE AND PROTEIN OF PNEUMOCOCCUS.

By OSWALD T. AVERY, M.D., and HUGH J. MORGAN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 8, 1925.)

In a previous paper on the immunological relationships of the cell constituents of Pneumococcus (1) two chemically distinct substances, namely the carbohydrate and protein of the cell, were shown to be intimately concerned in the serological specificity of this organism. The facts recorded at that time related only to the reactions exhibited by these substances in antipneumococcus serum prepared by immunization with the whole bacterial cell. In the absence of final evidence as to the antigenic properties of these two cell constituents the conclusions drawn were limited to their serological behavior. The observations made then have since been confirmed, and in the present report are extended to include the distinctive character of the isolated substances when each is tested for its function as antigen.

Antigenic Properties of the Isolated Carbohydrate of Pneumococcus.—The results of chemical studies previously reported and of those now in progress leave little doubt that the so called soluble specific substance of Pneumococcus of the three fixed types is in each instance a polysaccharide (2). The essential fact of significance here is that from pneumococci of Types I, II, and III, three different polysaccharides have been isolated, each chemically as distinct as it is serologically type-specific. The present work brings further proof that this important constituent upon which the type specificity of the cell depends is itself, when dissociated from other cellular substances, devoid of antigenic properties.

Attempts to immunize rabbits by injections of pneumococcus carbohydrate have invariably yielded negative results. Up to the pres-

ent time intravenous and cutaneous inoculations of these protein-free polysaccharides in considerable amounts and in repeated doses have failed to stimulate any demonstrable antibodies in the sera of animals so treated. This fact is of especial interest since in dilutions as great as 1:5,000,000 this non-protein, non-antigenic substance in purified form has been found specifically reactive in antipneumococcus serum of the homologous type.

Antigenic Properties of Isolated "Nucleoprotein" of Pneumococcus.—The protein material precipitated by dilute acetic acid from filtered solutions of pneumococci shows the chemical reactions of nucleoprotein and mucoid. It is realized that the product so obtained can scarcely be considered an antigenic unit, consisting as it necessarily does of a mixture of different proteins. Although the chemical procedures used in the isolation and purification of the protein may have caused some alteration in its chemical nature, this change is apparently not different from that involved in the mere process of cell dissolution, since the results with the isolated protein are identical with those obtained with simple extracts and solutions of cellular substances made by freezing and thawing cell suspensions (3).

The protein substance was prepared by the method given in a previous report (1). In some instances instead of dissolving the washed bacterial cells with the minimum amount of bile necessary to effect complete solution, concentrated suspensions of organisms in 0.002 N NaOH were subjected to repeated freezing and thawing to disrupt the cell bodies. This thick solution of dissolved organisms was then diluted with salt solution to one-tenth the volume of original culture fluid and passed through a Berkefeld V filter. To this filtered solution N/1 acetic acid was added slowly and the mixture carefully shaken, flocculation of the protein occurring promptly and completely at a reaction faintly acid to litmus. The precipitated protein was separated by centrifugation, washed several times in distilled water, and redissolved in salt solution by adding 0.1N NaOH until the solution was faintly alkaline to litmus. Freshly prepared protein solutions obtained in this manner were used for the tests since the previous method of drying the material in vacuum after rapid washing in acetone and ether rendered the substance less soluble. Solutions of pneumococcus protein prepared by this method exhibit the usual qualitative color reactions for substances of this nature: positive biuret, Hopkins-Cole, Millon, xanthoproteic, and Molisch reactions. The hydrolyzed protein gives the purine reaction with Fehling's solution. The protein solutions were standardized on the basis of their nitrogen content.

Rabbits were immunized by intravenous injection of pneumococcus nucleoprotein in faintly alkaline solution. The injections were given on 6 consecutive days followed by a free interval of 6 days. Three series of treatments were given. The daily dose for the initial course of injections averaged about 5 mg., the total amount of protein given during immunization varying from 0.1 to 0.2 gm.

The sera of animals injected with pneumococcus protein were tested for (*a*) agglutinins, (*b*) precipitins, and (*c*) protective antibodies.

(*a*) *Agglutinins.*—Antiprotein sera obtained by immunization of rabbits with pneumococcus nucleoprotein do not contain type-specific agglutinins for pneumococci. Antisera against the protein isolated from pneumococci of Types I and II and Group IV have in no instance agglutinated either the homologous or heterologous strains. The

TABLE I.

Absence of Precipitins for Pneumococcus Carbohydrate in Antiprotein Serum of Homologous Type.

Immune sera.	Carbohydrate of Pneumococcus Type II (Lot 17).				
	1:2000	1:20,000	1:40,000	1:80,000	1:160,000
Type II antiprotein serum.....	−	−	−	−	−
Type II antipneumococcus serum.....	++	+++	++++	++++	++++

−, no precipitation; ++, flocculation; ++++, compact disc-like precipitate.

absence of specific agglutinins in these sera is most strikingly demonstrated when saline suspensions of heat-killed organisms are used in the reaction. Under these circumstances the autolytic ferments are inactivated so that dissolution of the cell with the resulting liberation of its protein into solution is prevented. When unheated organisms are used in the test a slight, finely granular precipitate may be frequently observed after the reaction mixtures have stood overnight in the ice box. Since this reaction is always delayed and is non-specific, occurring without quantitative differences in the presence of both homologous and heterologous types of pneumococci, it seems reasonable to assume that its occurrence under these conditions is referable to the interaction of antiprotein serum with the protein freed by cell autolysis.

TABLE II.

Precipitins for Pneumococcus Protein in Antiprotein Sera of Homologous and Heterologous Types.

Immune sera.	Rabbit No.	Protein of Pneumococcus Type I.						Protein of Pneumococcus Type II.					
		1:2000	1:4000	1:8000	1:16,000	1:32,000	1:64,000	1:2000	1:4000	1:8000	1:16,000	1:32,000	1:64,000
Type I protein.	1	+	+	+++	++	++	−	+	++	+++	+++	++	±
	2	++	+++	++++	+++	++	+	+	++	++++	+++	+++	+
" II "	3	+	+++	+++	+++	++	±	\	\	+++	+++	++	±
	4	+++	++++	++++	+++	++	±	\	\	++++	+++	++	+
Normal.	Control.	−	−	−	−	−	−	−	−	−	−	−	−

±, faint cloud; +, slight finely granular flocculation; ++, moderate precipitation; +++, marked precipitation; ++++, complete precipitation easily broken up; \, not done.

The results of many experiments demonstrate that immunization with the acid-precipitable pneumococcus protein, free from cell fragments, does not induce specific agglutinins for the intact cell.

(*b*) *Precipitins.*—Just as antiprotein sera contain no agglutinins for pneumococci of the specific types, so also do they lack precipitins for the specific carbohydrate derived from these organisms (Table I). The stimulation of antibodies having these specific reactions seems to be a function of the whole bacterial cell, since neither the carbohydrate nor the protein separately possesses this particular antigenic property.

As the result of immunization with the nucleoprotein from any strain of Pneumococcus, antibodies are formed which react with the same

TABLE III.

Cross-Precipitin Reactions of Antiprotein Serum of Pneumococcus Group IV with Solutions of Pneumococci of Different Types.

Antiprotein serum of Pneumococcus Group IV.	Protein-containing solutions of pneumococci.				
	Dilutions of antigen.	Type I.	Type II.	Type III.	Group IV.
	1:20	++	++	+++	+++
	1:40	+	++	++	++
	1:80	−	+	+	+
	1:160	−	−	−	±

protein fraction of other types of pneumococci. In Table II evidence is presented that antisera against the proteins of Pneumococcus Types I and II cross-react in precipitin tests with the isolated protein of both types. Further evidence of this reciprocal action is found in Table III, which indicates that the serum of a rabbit immunized to the protein derived from Pneumococcus Group IV precipitates protein extracts of dissolved pneumococci not only of the homologous group but also of the three fixed types.

(*c*) *Protection.*—Whether or not antiprotein serum prepared by immunization with the isolated nucleoprotein alone confers passive protection in animals against infection must remain undecided until further evidence is accumulated. In one instance the serum of a rabbit immunized with the protein of Type I pneumococcus protected

mice against 0.001 cc. of homologous culture of which 0.000,001 cc. proved fatal for the untreated controls. With the other lots of sera, slight or no protection against infection with homologous or heterologous pneumococci was afforded.

More recent study indicates that antiprotein serum obtained by immunizing with filtered solutions of dissolved pneumococci, although yielding antibodies reactive with the protein, confers no passive immunity on mice infected with virulent pneumococci (3).

Although the evidence is not final as to the protective value of antiprotein sera, sufficient experimental data are available to warrant the conclusion that such sera at best are much less effective than the antipneumococcus sera obtained by immunization with the whole bacteria.

SUMMARY.

The data presented in this paper clearly indicate that the isolated carbohydrate and nucleoprotein constituents of Pneumococcus differ both serologically and antigenically one from the other. Moreover, each of these fractions of the cell separately exhibits immunological properties distinct from those manifested by the whole organism of which they form a part.

The carbohydrate is a protein-free polysaccharide and as such is devoid of the property of stimulating antibodies. Although in the free state, dissociated from other cellular substances, it is non-antigenic, in this form it still retains the property of reacting specifically in antipneumococcus serum of the homologous type. Further, this nonprotein constituent is not reactive with antiprotein serum. In other words, neither pneumococcus carbohydrate nor protein as separate antigen gives rise to antibodies with specific affinities for the carbohydrate or so called soluble specific substance of Pneumococcus.

The nucleoprotein of Pneumococcus, on the other hand, is antigenic. Immunization with this cell constituent gives rise to immune serum which precipitates solutions of pneumococcus protein without regard to the type from which it is derived.

The interrelations of the carbohydrate and protein of Pneumococcus as they exist in the intact cell to form the complete antigen, and the interpretation of the differences in the antigenic properties of the whole bacterium as contrasted with those of its component parts are reserved for discussion in a subsequent paper.

CONCLUSIONS.

1. The isolated carbohydrate of Pneumococcus is non-antigenic in the sense of stimulating antibody formation.

2. The isolated protein of Pneumococcus is antigenic: it induces the formation of antibodies which react with the nucleoprotein fraction of organisms of homologous and heterologous types.

3. The antiprotein sera do not agglutinate type-specific strains of Pneumococcus, or react with the carbohydrate derived from them.

BIBLIOGRAPHY.

1. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 81.
2. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73; 1924, xl, 301.
3. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1925, xlii, 355.

THE ANTIGENIC PROPERTIES OF SOLUTIONS OF PNEUMOCOCCUS.

By OSWALD T. AVERY, M.D., and JAMES M. NEILL, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 8, 1925.)

In the preceding paper (1) the immunological properties of intact pneumococci were compared with those of the isolated bacterial protein and carbohydrate. The whole cell, with antigenic functions unimpaired, gives rise to immune bodies which agglutinate pneumococci of the homologous type and precipitate the soluble specific substance, the carbohydrate, of the cell. It was shown that the bacterial protein stimulates the formation of precipitins which react with pneumococcus protein regardless of the type. This antibody to the protein does not agglutinate type-specific strains or precipitate solutions of the specific polysaccharide which distinguishes each type of Pneumococcus. The carbohydrate substance when dissociated from the bacterial cell has been found to possess no antigenic power; that is, in the free state it it is devoid of the property of inciting antibody formation. Antibodies reactive with the free carbohydrate can be produced only by immunization with intact organisms.

The subject of the present paper is the marked difference in the antigenic properties of intact pneumococci and the bacterial substances of these same organisms when in solution.

EXPERIMENTAL.

In the course of our previous studies on cellular oxidations by pneumococci, cell solutions or extracts of these organisms were prepared by a special method (2). These solutions, freed from all formed and living elements by Berkefeld filtration, were found to exhibit many of the physiological activities of the living cell. In addition to the active intracellular agents which function in cellular processes, they contain the constituents of the cell upon which its

immunological properties depend; namely, the pneumococcus carbohydrate and protein. Such cell solutions were employed, therefore, in the present study in order to compare the antigenic properties of dissolved pneumococci with those of the bacterial cells as such.

Methods.—Cell solutions of pneumococci were prepared by suspending the centrifuged bacteria from young broth cultures in small amounts of salt solution or culture fluid so that 1 cc. of the suspension contained the organisms from 50 cc. of the original broth culture. These concentrated cell suspensions were frozen and thawed repeatedly; the residual material was removed by centrifugation, and the supernatant fluid was passed through a Berkefeld filter. These extracts contain, therefore, no formed cell elements. They were proven sterile by cultural and mouse tests.

For purposes of comparative study, heat-killed suspensions of pneumococci were employed for immunization. The bacteria from 8 hour plain broth cultures were centrifuged and resuspended in salt solution in a volume equal to one-tenth that of the original culture. The bacterial suspensions were immediately heated in a water bath at 56°C. for 30 minutes. Young, actively growing cultures were used, and the bacterial suspensions were heated at once in order to minimize the amount of autolysis and cell solution which invariably result if such precautions are not observed. The reason for emphasizing these points will be apparent in the following experiments.

Immunization.—The sterile cell solutions were injected into rabbits intravenously on 6 consecutive days, followed by a free interval of 1 week. Four courses of injections were given, the doses gradually increasing from 0.1 cc. until a maximum of 2 cc. was administered daily in the last series of injections.

The suspensions of heat-killed bacteria were injected intravenously into rabbits in six daily doses of 0.1 cc. each, this amount of concentrated vaccine being suitably diluted to a volume of 1 cc. with salt solution at the time of injection. Four such courses of injections at weekly intervals were given, the animals receiving in all the equivalent of bacteria from 24 cc. of culture. The rabbits were bled on the 10th day after the last injection.

Tests.—In all precipitin reactions 0.2 cc. of immune serum, diluted to 0.5 cc. volume with salt solution, was added to an equal volume of graded dilutions of the substances to be tested, either the isolated carbohydrate or the protein fraction of the cell, or solutions of both substances as they occur in extracts of the original cells. Mixtures of the reacting substances were incubated for 2 hours at 37°C. The technique employed in agglutination and protection tests was the same as that described in detail in earlier papers.

In the following experiments the immune sera of rabbits injected with the cellular substances of Pneumococcus in solution and in the form of intact organisms were tested for the presence of (1) precipitins, (2) agglutinins, and (3) protective antibodies.

1. Precipitins for the Carbohydrate of Pneumococcus.—In order to determine whether or not rabbits immunized to solutions of pneumococci develop specific antibodies reactive with the soluble specific substance (carbohydrate), the sera of these animals were tested by the precipitin reaction against dilutions of this substance isolated in purified form from the three fixed types of pneumococci.

Repeated experiments have revealed the interesting fact that the sera of rabbits immunized with solutions of the cellular substances of pneumococci do not contain any demonstrable precipitins for the specific carbohydrate of the cell. A typical protocol is presented in

TABLE I.

Precipitins for the Type-Specific Carbohydrate of Pneumococcus in the Sera of Rabbits Immunized with Intact Organisms and with Solutions of the Cellular Substances.

Immune sera.	Specific carbohydrate isolated from Pneumococcus.						
	Type I.		Type II.			Type III.	
	1:1000	1:4000	1:4000	1:80,000	1:2,000,000	1:4000	1:80,000
Rabbit 1 (solutions of Pneumococcus Type II).....	−	−	−	−	−	−	−
Rabbit 2 (intact Type II pneumococci).....	−	−	+++	++++	++	−	−

++, turbidity; +++, turbidity and flocculation; ++++, rapid flocculation with compact sedimentation; −, clear, no reaction.

Table I. The presence of this carbohydrate antibody in bacterial immune sera, and its complete absence from the sera of animals immunized with solutions of the same cellular substances, indicate that the morphological integrity of the cell is essential for the development of its full antigenic power.

No matter whether dissolution of the bacterial cells is effected by the freezing and thawing process or by the lytic action of bile, the resulting solution, although containing both the carbohydrate and the protein substances of the whole bacterium, fails to stimulate the formation of the type-specific antibodies which react with the homologous

carbohydrate and which are always dominant in sera prepared by the injection of intact cells. The fact that the polysaccharide in the formed cell is specifically antigenic and that the same substance in cell solutions is non-antigenic forces the conclusion that the carbohydrate exists in the cell not merely as a free polysaccharide, but in combination with some other substance which confers antigenic properties upon it. With rupture of the cell and the passing into solution of its constituents this linkage is broken, and the free carbohydrate fraction, although still retaining the property of specific union with its type antibody, is itself no longer capable of inciting the formation of this same immune body. In summary, then, cell disintegration is accompanied by dissociation of the dominant antigenic complex, and the cell constituents thus dissociated exhibit immunological properties quite different from those characteristic of the formed cell.

2. Precipitins for the Protein of Pneumococcus.—The preceding experiments have shown that sera obtained by injection of solutions of the cellular substances contain no precipitins for the specific carbohydrate of the homologous type of Pneumococcus. In the following experiments the sera were tested for precipitins against the so called nucleoprotein isolated from pneumococci of homologous and heterologous types, and for their capacity to precipitate bacterial solutions containing, in addition to the carbohydrate, the cell protein unchanged by chemical treatment.

"Nucleoprotein," precipitated from solution by dilute acetic acid in the cold, was prepared from dissolved pneumococci of Types I and II and Group IV. This material, while subject to the changes incident to denaturation and representing a mixture of mucoid and other proteins, is referred to as nucleoprotein not to imply its exact nature, but to distinguish it from the protein-free carbohydrate fraction of the cell. Both of these cellular substances were separately tested in varying concentration against a constant amount of immune serum. The protein solutions were used in dilution of 1:400 and 1:4000, and the carbohydrate was employed in optimum concentration to avoid prozone inhibition.

Many experiments similar to that outlined in Table II have demonstrated the fact that animals immunized with dissolved pneumococci containing both the carbohydrate and protein of the cell in solution develop in their serum an antibody which is reactive with only one of

these substances; namely, the protein. Furthermore, whether produced as in the present instance by immunization with cell solutions or by the injection of the isolated protein alone (1), antiprotein sera react with pneumococcus protein regardless of its type derivation. Equally striking is the fact that although the material used for injection includes both the bacterial carbohydrate and protein, the sera thus developed are wholly devoid of any antibody reacting with the carbohydrate or type-specific substance of the cell.

Solutions of dissolved pneumococci, so far as their power to stimulate antibodies is concerned, behave precisely then as do solutions of pneumococcus protein alone. Although these solutions contain the

TABLE II.

Precipitins for the Protein and Carbohydrate Constituents of Pneumococcus Following Immunization with Cell Solutions of Type II Pneumococci.

Immune sera.	Nucleoprotein from Pneumococcus.				Carbohydrate from Pneumococcus.		
	Type I.	Type II.	Group IV.		Type I.	Type II.	Type III.
	Strain N.	Strain L.	Strain F.	Strain S.	Strain N.	Strain L.	Strain A.
Rabbit 1 (solutions of Pneumococcus Type II)	++	++	++	++	−	−	−
Normal rabbit	−	−	−	−	−	−	−

++, marked precipitation; −, no reaction.

two immunologically important constituents of Pneumococcus, the free carbohydrate when liberated from the cell, like the chemically purified polysaccharide itself, possesses no antigenic power. This fact is the more interesting since the carbohydrate in these cell solutions has been subjected to no chemical treatment such as is used in the preparation of the purified substance. When present in its free, dissociated state the carbohydrate neither functions as antigen nor participates in the protein precipitin reaction. That it does not function as antigen is shown by the fact that these sera are wholly devoid of the power of reacting with the type-specific carbohydrate (see Table II). That it does not participate in the protein reaction is proved by the fact that removal of the protein from cell solutions by

specific absorption leaves the carbohydrate free in the supernatant undiminished in titer.

Since antisera prepared by immunization with the cellular substances in soluble form never contain antibodies for the specific carbohydrate, while sera obtained by the injection of intact bacteria always show the presence of this antibody, it at once becomes obvious that the nature of the immune response is dependent upon the character of the material used for immunization. Because of the readiness with which Pneumococcus undergoes autolysis, suspensions of originally intact cells almost invariably contain not only formed elements but also more or less of the dissociated cell constituents in solution. Under these circumstances, antibacterial sera may and as a rule do exhibit not only the dominant type-specific carbohydrate-reacting antibody, but also in minor degree the protein antibody. The presence of this latter antibody is an expression of the difference in the antigenic properties of the same substances as they exist in the whole, complete cell, and in the dissociated state following cell disintegration. This fact is brought out in Table III, in which the serum of a rabbit immunized with suspensions of originally intact pneumococci is shown to exhibit slight reactions with the common protein in the cell solutions of heterologous types.

Table III also emphasizes the fact that immunization with cell solutions of pneumococci of one type evokes antibodies which precipitate solutions of organisms of all other types. Since immune sera prepared in this manner have been found to be reactive only with the protein and to be wholly devoid of the property of interacting with the carbohydrate of the cell, it is evident that this precipitating action is due to the presence of an antibody reactive with the protein common to all pneumococci.

For reasons already defined the serum of an animal immunized with suspensions of pneumococci may show in addition to the distinctive type-specific carbohydrate reaction a slight antiprotein reaction when tested against the protein-containing solutions of other types. The presence of a certain amount of this protein antibody in antibacterial sera, in the preparation of which supposedly only intact cells have been used, is to be expected in view of the readiness with which pneumococci undergo dissolution in the test-tube and probably also in the animal body.

The facts to be emphasized here are that when filtered solutions or extracts of bacterial substances free from all formed elements are used in immunization, there results no antibody which is reactive with the specific carbohydrate of the cell; secondly, that this type of antibody, as far as our experimental knowledge goes, is produced only by immunization with intact cells.

3. Agglutinins.—In comparing the differences in agglutinin response to immunization with pneumococci in the dissolved and formed

TABLE III.

Comparison of the Common Protein and Type-Specific Carbohydrate Precipitins Present in Immune Sera of Rabbits Immunized with Cell Suspensions and Cell Solutions of Pneumococcus Type II.

Sera of rabbits immunized with	Cell solution of pneumococci containing protein and carbohydrate of								
	Type I.			Type II.			Type III.		
	1:2	1:20	1:200	1:2	1:20	1:200	1:2	1:20	1:200
Solutions of Type II pneumococci.	++++	+++	+	++++	+++	+	++++	+++	+
Intact Type II pneumococci.....	+	±	−	xxxx	xxxx	xxxx	+	±	−

Protein reactions: ±, faint cloud delayed; +, turbidity, no precipitate; +++, turbidity and granular precipitation; ++++, marked precipitation.

Carbohydrate reaction: xxxx, immediate flocculation settling as compact disc.

The protein-antiprotein reaction varies from a cloudiness to a finely granular precipitate which is easily broken up on shaking the tube; the anti-*S* or carbohydrate reaction is characterized by a rapidly appearing, coarser flocculation which settles to the bottom of the tube as a firm, compact disc not easily broken up on agitation.

state, the sera of rabbits immunized against single strains of different types were studied. Since the nature of the reactions was identical in all cases, a type protocol of an individual serum is given in Table IV.

Cole (3) in 1914 observed that sera obtained by the immunization with bile solutions of pneumococci contain no agglutinins for the homologous type of organism. This observation is confirmed in the present study, in which the absence of specific agglutinins in the sera of rabbits treated with cell solutions has been constantly noted.

Just as these bacterial solutions, when used as antigen, fail to stimulate the formation of precipitins for the specific, homologous carbohydrate of the cell, so do they lack the property of inciting specific agglutinins for the intact organisms. Since in the case of Pneumococcus, agglutination is considered to be the result of an interaction at the periphery of the cell between the type-specific carbohydrate and its homologous antibody, the same reasons which account for the absence of the carbohydrate precipitins will explain the absence of specific agglutinins.

4. Protective Antibodies.—Rabbits were immunized with heat-killed pneumococci by the method described by Cole and Moore (4). The sera of animals which received relatively small amounts of intact bacteria afforded specific protection against 100,000 times the

TABLE IV.

Agglutinins in Rabbit Serum Following Immunization with Cell Solutions and Cell Suspensions of Pneumococcus Type II.

Serum of rabbits immunized with	Saline suspensions of pneumococci.*		
	Type I.	Type II.	Type III.
Solutions of Pneumococcus Type II	−	−	−
Intact Type II pneumococci	−	++++	−
Normal rabbit	−	−	−

−, no agglutination; ++++, complete agglutination.

* The bacterial suspensions were heated for 40 minutes at 56°C. to prevent autolysis of the cell.

dose of virulent organisms which was invariably fatal for control mice. On the other hand, the sera of rabbits injected with concentrated solutions of the dissolved bacteria were without protective value. Sera of this type, as pointed out in connection with the preceding experiments, contain antibodies reactive only with pneumococcus protein, and possess neither agglutinins for the formed cells nor precipitins for the soluble specific carbohydrate derived from them. The principles underlying the protective mechanism of anti-pneumococcus serum are not as yet sufficiently understood to relate this phenomenon to the presence or absence of any particular antibody. The data presented indicate clearly that the dissociated substances of dissolved pneumococci are not capable of eliciting the full protective

response stimulated by the bacterial cell as a whole. Just as antibodies reactive with the bacterial cell *in vitro* are evoked only when intact organisms are used as antigen, so antibodies conferring passive protection against pneumococcus infection are stimulated most readily and most effectively by immunization with the whole cell.

DISCUSSION.

It is not the purpose of the present discussion to attempt or suggest an interpretation of the experimental data in terms of their more general immunological significance. But for pneumococci, at least, they serve to point out striking differences in the antigenic properties of the bacterial substances as they exist in the cell itself and as they occur in solutions of the dissolved organisms. This difference consists in the loss of a specific antigenic function which the cell suffers whenever dissolution occurs. The function lost is the capacity to stimulate the formation of the type-specific antibodies which characterize the immune response to the uninjured bacterial cell. Antibacterial sera containing these antibodies specifically agglutinate pneumococci of the homologous type and precipitate the free carbohydrate derived from bacteria of the corresponding type. On the other hand, solutions of the same organisms containing both the carbohydrate and protein of the original cell, but free of all formed elements, fail to induce antibodies having either of these specific properties. Yet solutions of dissolved bacteria, although lacking the complete antigen of the whole cell, are not without antigenic properties. By reason of the protein they contain, these solutions induce antibodies which react with pneumococcus protein, and by virtue of the common character of this constituent in pneumococci, sera containing the protein antibody cross-react with protein from all types of pneumococci. The dominant antigenic character of the intact cell is its capacity to evoke the type-specific carbohydrate-reacting antibody, while the same cells in solution yield only the protein antibody of the species. This protein-antiprotein reaction is not limited by type differentiation, and, as Lancefield (5) has recently shown, extends to the more or less closely related cocci. Antiprotein sera obtained by immunization with dissolved pneumococci are similar in

their immune reactions to sera produced by injection of the isolated protein alone; that is, they react with pneumococcus protein regardless of its type derivation and they fail to react with the free carbohydrate or with the intact cells of the homologous type.

Previous studies from this laboratory (6) have shown that it is the carbohydrate constituent, the so called soluble specific substance of Pneumococcus, which endows the bacterial cell with type specificity. The work of Zinsser, Mueller, and their associates (7) indicates quite clearly that similar relationships hold for a number of other bacteria and yeast. Since the carbohydrate of Pneumococcus when dissociated from the cell, although still retaining its capacity to unite with antibody, loses its antigenic power, one may assume that in the intact organism this constituent exists not only as free carbohydrate, but also in combination with some other substance which confers upon it true antigenicity. What the nature of this antigenic union is, whether it is one between the carbohydrate and protein of the cell or not, is as yet undetermined. The evidence presented in the present paper indicates, however, that when the cell undergoes dissolution the specific antigenic complex is dissociated, and that the resulting solution, containing both the cell protein and carbohydrate in the free form, exhibits only those antigenic properties due to the common protein. It is not surprising, therefore, that in antipneumococcus sera there is commonly present not only the dominant type-specific antibody, but to a greater or less extent the protein antibody of the species. The presence of the latter antibody in any given lot of serum is, upon the bases of this analysis, referable to the amount of cell dissolution which has taken place more especially before and possibly to some extent after injection of the organism into the animal body.

CONCLUSIONS.

1. Intact pneumococci, possessing specific antigenic powers unimpaired by cultural or other procedures, give rise to agglutinins for organisms of the homologous type and to precipitins for the type-specific carbohydrate derived from them.

2. Solutions of pneumococci free of all formed elements, but containing the carbohydrate and protein of the original cell, fail to stimu-

late the formation of type-specific antibodies. Sera prepared in this manner do not react with the carbohydrate constituent of the cell and do not agglutinate organisms of the homologous type. The loss of this antigenic function is related to changes incurred during dissolution of the bacterial cell.

3. Solutions of the cellular substances of Pneumococcus, although lacking the specific antigen of the whole cell, induce the formation of antibodies reactive with pneumococcus protein regardless of the type from which the latter is derived.

BIBLIOGRAPHY.

1. Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1925, xlii, 347.
2. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 347, 357, 543, 745.
3. Cole, R., *J. Exp. Med.*, 1914, xx, 346.
4. Cole, R., and Moore, H. F., *J. Exp. Med.*, 1917, xxvi, 537.
5. Lancefield, R. C., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 109.
6. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 81. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1924, xl, 301.
7. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275. Mueller, J. H., and Tomcsik, J., *J. Exp. Med.*, 1924, xl, 343. Zinsser, H., and Mueller, J. H., *J. Exp. Med.*, 1925, xli, 159. Mueller, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 209. Mueller, J. H., Smith, D. E., and Litarczek, S., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 373.

IMMUNOLOGICAL RELATIONSHIPS OF CELL CONSTITUENTS OF PNEUMOCOCCUS.

Second Paper.

By OSWALD T. AVERY, M.D., and MICHAEL HEIDELBERGER, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 8, 1925.)

In the course of studies carried on in this laboratory during the past 3 years on the chemical and immunological properties of the cell constituents of Pneumococcus, certain facts have been acquired which indicate a significant relationship between the chemical constitution and biological specificity of the bacterial cell.[1,2] It seems desirable at this time to bring together in summary form these facts in order to relate them to the problem as a whole.

In the work thus far, two of the cellular constituents of Pneumococcus have been studied. One of these is the substance precipitated from solutions of Pneumococcus by dilute acetic acid, which, although comprising a mixture of proteins, may for the purposes of the present discussion be referred to as bacterial nucleoprotein. The other constituent, non-protein in character, is the so called soluble specific substance, which is now considered to be a carbohydrate of the polysaccharide type. While it is realized that these two substances, important as they are, do not comprise the whole antigenic mosaic of the cell, they are emphasized at this time because they happen to be the first chosen for investigation, and the only ones thus far isolated.

Studies of the antigenic and serological properties of these two chemically diverse substances as they exist together in the intact cell and free in a dissociated state, have yielded certain facts which contribute to a better understanding of the differential specificity of the

[1] For the first paper see Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 81.

[2] *Cf.* in part the two preceding papers, also the chemical studies from this laboratory referred to in these.

various types of pneumococci. For not only do these cellular compounds belong to two wholly different classes of chemical substances namely protein and carbohydrate, but the carbohydrate constituent of each of the three fixed types of pneumococci has been found to be a chemically different polysaccharide. These substances possess the unique distinction of reacting specifically with antibacterial serum of the homologous type, although in the free state, dissociated from the cell, they are devoid of the power of inciting the formation of antibodies upon injection into animals. The protein, on the other hand, is antigenic; the serum of an animal immunized with this substance reacts with protein derived from any type of Pneumococcus. It at once becomes obvious that as these two components exist in the cell, they enter into an antigenic complex which has different immunological properties from those exhibited by either substance alone. The serological differentiation of the various types of pneumococci is dependent, therefore, not only on the chemical and antigenic differences in these component substances, but is also intimately related to the structural character and morphological integrity of the bacterial cell.

That these principles, so strikingly exemplified in the immunological reactions of Pneumococcus, are of more general biological significance is illustrated in the somewhat analogous relationships existing within other groups of bacteria and of yeasts. The work of Zinsser, Mueller, and their associates (1) indicates that from a number of microorganisms, such as staphylococci, meningococci, and Friedländer, typhoid, and tubercle bacilli, a non-protein residue substance may be extracted which bears a definite relation to the specific character of the bacteria-cell. Smith and Reagh (2) in 1903 described differences in the serological behavior of motile and non-motile bacilli of the hog-cholera group. Recently Orcutt has shown that these differences are dependent upon two separate agglutinogens corresponding to the flagellar and somatic substances of the cell; the presence of both of these factors in the motile forms and the absence of the flagellar antigen in non-motile types account for the variations observed in the immunological behavior of these bacilli. Felix (3) and others have found that the differences in agglutination of the O and H forms of *proteus* bacillus are related to the occurrence in these organisms of one or both of

two separate substances, involving the endo- and ectoplasm of the cell. These analogies while close are not absolute; the flagellar substance of motile bacilli is in itself antigenic, while the soluble specific substance of Pneumococcus is a non-antigenic carbohydrate. The significant fact, however, is that in these widely divergent groups of microorganisms two distinct cellular substances are determinative factors in bacterial specificity.

Structure of the Cell.—Before considering in detail the immunological characters of these two cellular constituents of Pneumococcus it may add to the clearness of the discussion to picture the form or pattern of the cell as it relates to the disposition of these substances. For undoubtedly cell configuration reflects in some measure the ease with which this organism participates in immunity reactions and the avidity with which it interacts with antibody. Many of these reactions are presumably surface phenomena, and the nature of the reactive material at the periphery of the cell may determine the readiness of response and even the specificity of reaction. Pneumococcus is an encapsulated organism, and there are grounds for the belief that the ectoplasmic layer of the cell is composed of carbohydrate material which is identical in all its biological characters with the type-specific substance of Pneumococcus. On the other hand, the endoplasm, or somatic substance, consists largely of protein which, as previously pointed out, is species- and not type-specific. This protein is possessed in common by all pneumococci while the carbohydrate is chemically distinct and serologically specific for each of the three fixed types. The cell, therefore, may be conceived of as so constituted that there is disposed at its periphery a highly reactive substance upon which type specificity depends. The structure and, as will be pointed out later, the morphological integrity of the cell are determinative factors in bacterial specificity.

Chemistry of the Soluble Specific Substance.—The following data briefly summarize our knowledge of the soluble specific substance of the three fixed types of Pneumococcus at the stage of purification so far attained (4).

The soluble specific substance of Type II pneumococcus appears to be a *weakly* acidic, nitrogen-free polysaccharide made up chiefly of glucose units. Its specific optical rotation is about *plus* 74°. It

reacts at a dilution of 1:5,000,000 with antibacterial serum of Type II pneumococcus, and does not react with Type I or Type III antisera.

The soluble specific substance of Type III pneumococcus, while also apparently a nitrogen-free polysaccharide, differs from the Type II derivative in many particulars. It rotates the plane of polarized light about 33° to the *left*. It is a *strong* acid and is made up of units of glucose and either glucuronic acid or some derivative of this acid. It also separates in insoluble form from solutions strongly acidified with hydrochloric acid. In as high a dilution as 1:6,000,000 it still reacts with Type III antipneumococcus serum.

TABLE I.

Chemical Characteristics of the Soluble Specific Substances of Types I, II, and III Pneumococcus.

Type.	Optical rotation.	C	H	N	Acid equivalent.	Reducing sugars on hydrolysis.		Highest dilution giving precipitate with homologous immune serum.
						per cent		
I	+300°	43.3*	5.8	5.0†		28	(Galacturonic acid.) (Amino sugar derivative.)	1:6,000,000
II	+74°	45.8	6.4	0.0	1250	70	Glucose.	1:5,000,000
III	−33°	42.6	5.6	0.0	340	75	Glucose (glucuronic acid).	1:6,000,000

* Theory for $(C_6H_{10}O_5)_x$: C = 44.4 per cent; H = 6.2 per cent.
† Amino N: 2.5 per cent.

The Type I soluble specific substance (4), on the other hand, while also polysaccharide in nature, differs from the other two type-specific substances in containing nitrogen as an apparently essential component. In spite of a nitrogen content of 5.0 per cent the substance gives none of the usual protein color tests. One-half of the nitrogen is liberated when the substance is treated with nitrous acid. Reducing sugars appear at the same time and the specific reaction vanishes, and since the carbon and hydrogen content are close to the theoretical values for polysaccharides it appears likely that a nitrogenous sugar derivative is involved. It is a strong acid and a weak base, and is very sparingly soluble in water at the isoelectric point. Its specific optical rotation is +300°, and on oxidation with nitric acid it yields mucic

acid. In the specific precipitin reaction with homologous antipneumococcus serum it can be detected in dilutions as great as 1:6,000,000.

In Table I are summarized the available data concerning the chemical differences in the polysaccharide derivatives of pneumococci of Types I, II, and III.

Although the specific polysaccharide by itself evokes no antibodies upon injection into rabbits it is specifically reactive with antibody induced by immunization with intact cells. The fact that this specifically reactive carbohydrate is non-antigenic when dissociated from the other cellular constituents and is capable of inciting antibody formation only in the form in which it is present in the intact cell, forces the conclusion that in the latter instance it exists not merely as free carbohydrate but also in combination with some other substance which confers upon it specific antigenic properties. Immunization with intact bacteria containing this carbohydrate complex elicits antibodies which not only agglutinate the formed cells but precipitate solutions of the carbohydrate isolated from pneumococci of the homologous type. How the specific polysaccharide is combined in the cell, whether with protein or some other constituent is not yet clear, but it is evident that the compound thus formed is the dominant and essential antigen of the cell, and the one responsible for type specificity.

The immunological relationships of the protein and carbohydrate fractions of the cell are graphically presented in Fig. 1, in which *S* represents the specific soluble substance (carbohydrate) and *P* the protein of the Pneumococcus. The symbols used are in no sense to be construed as interpretive of the mechanism involved, but serve simply to visualize the interaction between these cell constituents and their respective antibodies. The reactions illustrated may be briefly summarized as follows:

A (*Fig. 1, A*).—Immunization with intact cells of a given type of Pneumococcus gives rise in the serum to the presence of antibodies which are type-specific; such sera specifically agglutinate the homologous type of Pneumococcus, protect mice against virulent organisms of the same type, and precipitate solutions of the corresponding purified carbohydrate (Table II). While this type-specific carbohydrate antibody is provoked only in response to the antigenic stimulus of the whole cell, a serum containing this antibody alone without a trace of

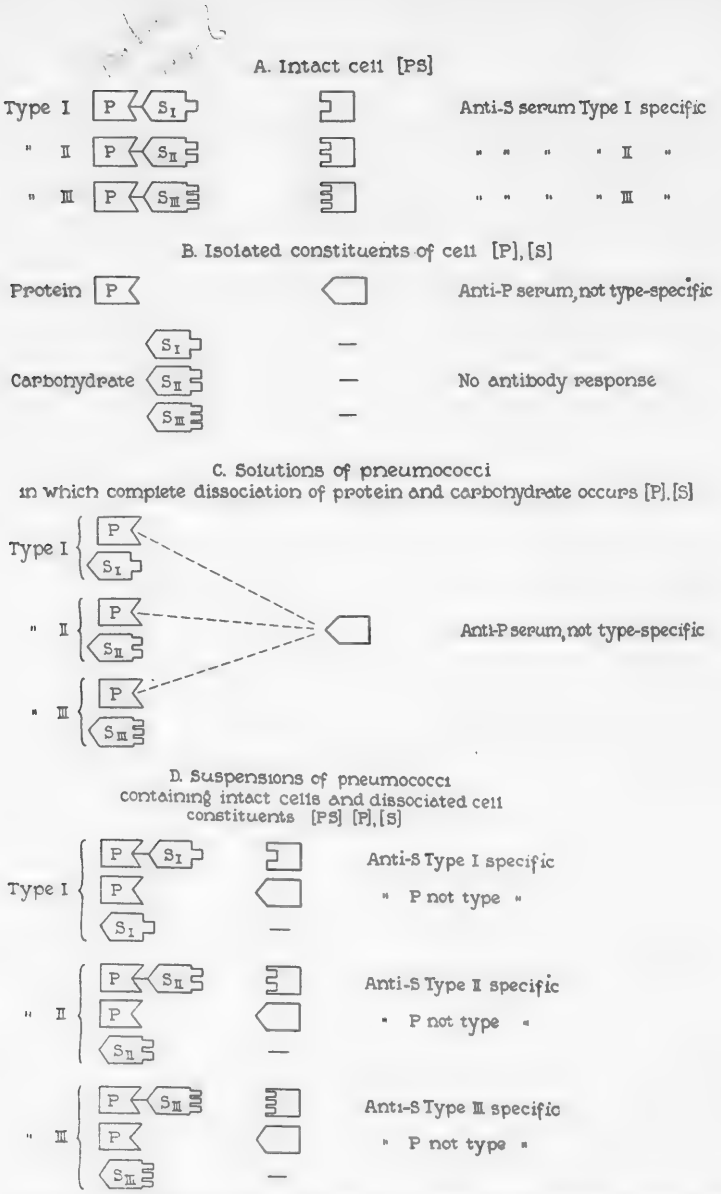

A. Intact cell [PS]
Type I
Anti-S serum Type I specific
B. Isolated constituents of cell [P], [S]
Protein
Anti-P serum, not type-specific
Carbohydrate
No antibody response
C. Solutions of pneumococci
in which complete dissociation of protein and carbohydrate occurs [P], [S]
Anti-P serum, not type-specific
D. Suspensions of pneumococci
containing intact cells and dissociated cell
constituents [PS] [P], [S]
Anti-S Type I specific
P not type
Anti-S Type II specific
Anti-S Type III specific

FIG. 1. Immunological relationships of protein and soluble specific substance (carbohydrate) of Pneumococcus.

protein antibody is rarely obtained for reasons which will be discussed later. Whether singly or in conjunction with other antibodies this specific carbohydrate antibody has been observed only under conditions in which the intact bacterial cell has been employed for immunization.

B (*Fig. 1, B*).—The specific polysaccharide (*S*) when isolated from the cell is incapable of inciting antibody formation upon injection into

TABLE II.

Pneumococcus and cell constituents		Antibodies demonstrable in serum						
Material used for immunization	Effective antigen	Agglutinins	Precipitins		Complement fixation		Specificity	
			S	P	S*	P	Type	Species
Intact cells [SP]†	[SP]†	+	+	−	+	−	+	−
Carbohydrate S‡	None	−	−	−	−	−	−	−
Protein P‡	P	−	−	+	−	+	−	+
Solutions, extracts containing free S and free P	P	−	−	+	−	+	−	+
Suspension of intact cells and dissociated cell constituents [SP], free S, free P	[SP], P	+	+	+	+	+	+	+

†[SP] = Carbohydrate and protein combined antigen of cell

‡S = Free carbohydrate, the soluble specific substance of cell

‡P = Free protein of cell

*Free S, as antigen, does not fix complement with immune horse serum; is active with immune rabbit serum[3]

animals. The isolated protein (*P*), on the other hand, is antigenic and gives rise to an immune serum which reacts with pneumococcus protein regardless of the type from which it is derived. Antiprotein sera do not agglutinate type-specific strains of pneumococci and do not precipitate solutions of the soluble specific substance (*S*) (Table II).

C (*Fig. 1, C*).—Solutions and extracts of pneumococci behave antigenically precisely as do solutions of pneumococcus protein; the disso-

[3] *Cf.* Vollmond, E. (in press).

ciation of the antigenic complex which occurs whenever the cell is dissolved results in the liberation of free carbohydrate (*S*) and free protein (*P*) in solution. In such solutions and extracts the only constituent which functions as antigen is the protein, for free *S* is non-antigenic. The sera of animals immunized with solutions of pneumococci, in which complete dissociation of these cell constituents has taken place, contain only antibodies reactive with the protein. Such sera exhibit the same reactions as do those prepared by immunization with protein alone (Table II).

It is evident from these facts that morphological dissolution of pneumococci is accompanied by antigenic dissociation, for sera prepared from filtered solutions of disintegrated cells free of formed elements fail to exhibit any of the dominant type-specific properties which characterize sera obtained by immunization with whole bacteria (compare *A* and *C*, Fig. 1). Morphological integrity of the bacterial cell, therefore, is requisite for the expression of its full antigenic power.

D (*Fig. 1, D*).—It becomes obvious, therefore, that the character of the antibody response is determined by the nature of the cell material used for immunization. The injection of suspensions of pneumococci into animals induces the formation of antibodies against *S* alone or against both *S* and *P* separately, depending upon whether or not these suspensions contain only intact cells or a mixture of both intact and dissolved cell bodies. Since pneumococci readily undergo autolysis and dissolution, suspensions and indeed cultures of these organisms almost invariably contain not only formed elements, but also more or less of dissociated cell constituents in solution. The predominance of the former in such suspensions stimulates the production of the type specific *S* antibodies, while the occurrence in these same suspensions of dissociated cell protein provokes the formation of the species-specific protein antibodies. The more common result of immunization with cell suspensions is the occurrence in the serum of both the carbohydrate and protein antibodies. The former invariably predominate, the latter are present in varying concentration depending upon the amount of cell dissolution which has taken place in the material previous to or after injection into the animal body. Therefore, use of suspensions of pneumococci containing both intact cells and the soluble products of cell disintegration yields on immunization not only type-

specific antibodies but antibodies reacting with the protein substance which is common to all pneumococci. It is the presence of this protein antibody with its broader zone of activity which is responsible for the confusing cross-immunity reactions occasionally encountered in supposedly type-specific sera. That these two antibodies are separate and distinct is shown by absorption tests; the antiprotein reacting bodies in such sera can be removed by absorption with the protein of a heterologous type without diminishing the titer of specific agglutinins for the homologous culture or the precipitins for the specific polysaccharide of the corresponding type.

The relationship of the carbohydrate constituent of Pneumococcus to type specificity, and the establishment of its identity with the soluble specific substance previously discovered (5) in culture fluids and in the urine and blood of patients suffering from pneumococcus pneumonia are facts indicative of the biological importance of this substance both to the bacterial and animal organisms.

The elaboration of this specific carbohydrate is a specialized function most active in pneumococci rapidly multiplying in the animal body or in suitable culture media. The stimulus to the full expression of this function is found under circumstances providing optimal growth conditions. Under these conditions pneumococci exhibit maximal capsular development, exalted virulence, and distinct type specificity. Conversely, when optimal conditions are lacking the organisms may become degraded, lacking capsules, of little or no virulence, and devoid of type distinction. Without further supporting evidence it would of course be hazardous to venture the assertion that all three of these characters are necessarily and causally related to the *S*-producing function of the cell. However, the earlier work of Stryker (6) and the more recent studies of Griffith (7), Reimann (8), and Amoss (9), as well as our own observations, indicate that under certain cultural environments pneumococci lose their type specificity and become avirulent. Moreover, these changes are now known to be accompanied by a loss of the capacity to elaborate this soluble specific substance.

In 1917 Cole (10) observed that infected exudates and sera containing these soluble reactive substances of pneumococci possessed the property of neutralizing pneumococcus antibodies, and pointed out the

significance of this fact in relation to the therapeutic administration of immune serum. Moreover, the work of Sia (11) on the effect of this carbohydrate substance on the growth of pneumococci in normal serum-leucocyte mixtures indicates that the addition of this substance in very small amounts exerts a definite and specific effect in annulling the inhibitory action of the sera of naturally resistant animals.

If final proof be brought for the conception that the capsular zone of the organism is largely composed of this carbohydrate substance, is part of the defense mechanism of the cell, and is the site of its initial contact with antibody, then these soluble bacterial polysaccharides acquire new significance not only in the serological reactions of the cell, but in the actual processes of infection and immunity in the host.

SUMMARY.

In this paper the general immunological significance of the intact pneumococcus cell and of its protein and carbohydrate components is discussed.

BIBLIOGRAPHY.

1. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275. Mueller, J. H., and Tomcsik, J., *J. Exp. Med.*, 1924, xl, 343. Zinsser, H., and Mueller, J. H., *J. Exp. Med.*, 1925, xli, 159. Mueller, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 209. Mueller, J. H., Smith, D. E., and Litarczek, S., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 373.
2. Smith, T., and Reagh, A. R., *J. Med. Research*, 1903, ix, 270.
3. Felix, A., *J. Immunol.*, 1924, ix, 115.
4. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, xlii (in press).
5. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.
6. Stryker, L. M., *J. Exp. Med.*, 1916, xxiv, 49.
7. Griffith, F., *Rep. Pub. Health and Med. Subj., No. 18*, 1923, 1.
8. Reimann, H. A., *J. Exp. Med.*, 1925, xli, 587.
9. Amoss, H. L., *J. Exp. Med.*, 1925, xli, 649.
10. Cole, R., *J. Exp. Med.*, 1917, xxvi, 453.
11. Sia, R. H. P., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 262.

THE IMMUNOLOGICAL RELATIONSHIPS OF STREPTOCOCCUS VIRIDANS AND CERTAIN OF ITS CHEMICAL FRACTIONS.

I. Serological Reactions Obtained with Antibacterial Sera.

By REBECCA C. LANCEFIELD.

(*From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, and the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 22, 1925.)

This analysis of the reactive and antigenic substances obtainable from non-hemolytic streptococci was begun as a result of previous work which showed the presence in the serum of certain rheumatic fever patients of complement binding antibodies for streptococcus extracts. The earlier work, to be reported in detail elsewhere, showed that positive reactions were obtained in a variety of diseases when nucleoprotein extracts of *Streptococcus viridans* cultures were used as antigens. These cross-reactions indicated the desirability of investigating the entire antigenic complex of *Streptococcus viridans* in a manner similar to that employed by Avery and Heidelberger (1–3) in their work on pneumococcus. As a preliminary to a satisfactory evaluation of the importance of certain types of streptococcus antibodies in the serum of rheumatic fever patients, it was necessary to know what antibodies an antistreptococcus serum might contain. This could best be determined by a study, on the one hand, of the reactive and antigenic substances present in cultures and in extracts of non-hemolytic streptococci, and, on the other hand, by a study of the antibodies evoked in rabbits in response to immunization with cultures and their chemical fractions. It seemed desirable also to ascertain whether the conclusions of Avery and Heidelberger in regard to pneumococcus were applicable to another species of bacteria, or whether there would be discovered in a heterogenous group, such as the green streptococci, a set of conditions different from those existing in a species with sharply defined types.

In the work referred to, Avery and Heidelberger (2) found a nucleoprotein fraction common to all types of pneumococci. The sera of animals immunized with purified nucleoprotein from one type gave equally good reactions with protein from any other type. Type specificity, however, was found due to the presence of the soluble specific substance of Dochez and Avery (4) which has recently been subjected to intensive chemical and immunological analysis by Heidelberger and Avery (1, 3). This substance is a complex carbohydrate which is chemically, as well as immunologically, distinctive for each type of pneumococcus, and is serologically active in very high dilutions. In contrast to the antigenic *species-specific* nucleoprotein, this *type-specific* carbohydrate has never been found to incite the production of antibodies after separation from its cell complex.

A number of other investigators have reported highly reactive specific substances present in culture filtrates or in extracts of bacterial sediments. In a few instances such substances have also been found in the blood and urine of patients, as well as in the body fluids of experimental animals. Fornet (5) in 1906 found precipitable substances in the blood and urine of typhoid fever patients and in the blood of experimentally infected rabbits. Ascoli (6), and Hamburger (7) as well, had shown that precipitinogen passed through the kidney and could be demonstrated in the urine. Dochez and Avery (4) in 1917 isolated type-specific substances from young actively growing pneumococcus cultures and from the blood and urine of pneumonia patients and of pneumococcus-infected rabbits.

Specific precipitable substances in culture filtrates have been studied extensively since the early work of Kraus; but recently attention has been particularly focussed on substances obtained from culture filtrates or from extracts of bacterial or other cell bodies as being causally related to specificity. Thus Zinsser (8) and his associates (Parker (9), Mueller (10), Mueller and Tomcsik (11), Petroff (12)) have shown that "residue antigens" from a variety of bacteria, and also from yeast, are non-protein specifically reactive substances which are not antigenic in chemically purified condition, although they incite the production of antibodies while still in the unaltered state within the cell. The work in this field of Dochez and Avery and of Heidelberger and Avery has already been mentioned. Hitchcock (13) also has tested relatively protein-free precipitating substances from a number of non-hemolytic streptococci and has found an array of heterogenous strains by this method. He has also studied (14) substances from hemolytic streptococci which are apparently chemically similar to "residue antigens" from other bacteria but which differ serologically in that cross-reactions are obtained with extracts from immunologically distinct groups of hemolytic streptococci. The meningococci have been analyzed from the same point of view by Przesmycki (15) with similar results: a practically type-specific substance has been found for each group. Landsteiner, van der Scheer, and Witt (16) have demonstrated recently that alcoholic extracts of human red blood cells contain the specific reactive substances, probably lipoidal in nature, for the blood groups of man. Thus the substances responsible for specificity have been isolated in varying degrees of chemical purity from a number of bacterial species, from yeast cells, and from red blood cells.

A systematic immunological study of the less specific nucleoprotein fraction has been made only for pneumococcus. Avery and Heidelberger (2) have demonstrated that the chemically extracted nucleoproteins of pneumococcus show *species specificity* rather than *type specificity*. Furthermore, this fact has been related to certain instances of non-specific agglutination (17) in that pneumococci which have lost their capsules and the function to produce the specific substance show non-specific agglutination due to the uncovering of the protein contained in the bacterial bodies. Other authors have reported group reactions which are probably in a similar manner directly dependent on the presence of related proteins. Kinsella and Swift (18, 19) by means of the complement fixation reaction showed certain group relationships among the non-hemolytic streptococci and among the hemolytic streptococci as well. Hitchcock (20), using the same method, has recently reported complicated group relationships between the hemolytic and the non-hemolytic streptococci and the pneumococci. Meyer and Christiansen (21) on the basis of their study of cutaneous hypersensitiveness of rabbits to typhoidin and similar preparations believe that all bacteria must contain some protein substances in common. Eberson (22), after studying the effect of ultraviolet rays on the antigenic properties of meningococci, concluded that a single protein structure represents the element common to groups of biologically related organisms.

This review of the literature indicated that many bacteria contain a highly reactive specific substance which can be extracted chemically and that at least one species, pneumococcus, contains another fraction common to the entire species. Scattered observations were also found which tended to show, less conclusively than for pneumococcus, that the protein fractions of some other species were responsible for group reactions. It seemed important therefore to determine the basis of group and specific reactions of the non-hemolytic streptococci both from the point of view of their possible connection with rheumatic fever and from a more general consideration of the immunological relationships involved.

Methods.

Four strains of *Streptococcus viridans* were selected. Of these, two (A49 and 38D) were isolated by Dr. H. F. Swift from blood cultures of rheumatic fever patients and two were obtained from blood cultures in cases of bacterial endocarditis. All were methemoglobin producers, were bile-insoluble and had the fermentation properties recorded in Table I. Various other strains were used incidentally, but these four were most carefully studied.

Rabbits were immunized with from three to six doses of living broth cultures

injected intravenously twice a week. The first dose was 3 to 5 cc. with subsequent doses usually double the preceding one until a total of 20 cc. was reached; by this time the animals were usually highly immune; they were bled from 5 to 12 days after the last injection.

The materials were extracted from the bacteria according to Woolridge's (23) method for the nucleoproteins and Heidelberger and Avery's (1, 3) method for the soluble specific substance, with certain modifications required for this kind of organism. From 3 to 12 liters of an 18 hour plain broth culture were centrifuged, the bacterial sediment put into a flask with about 2 cc. of sterile sand, and dried *in vacuo* at a temperature not exceeding 37°C. This dry mass was then ground in a ball mill to a fine powder in from 1 to 7 days. Then 100 cc. of N/100 sodium hydroxide were added for every 3 liters of original culture and the flask agitated overnight in the cold. The suspension was centrifuged at high speed, and the sediment of sand and bacterial detritus discarded. The opalescent supernatant

TABLE I.

Fermentation Reactions of the Four Strains Chiefly Studied.

Strain.	Milk.	Raffinose.	Inulin.	Salicin.	Mannitol.	Lactose.	Saccharose.
W67	+	+	−	+	−	+	+
A148	+	+	−	−	−	+	+
A49	+	−	−	−	−	+	+
38D	+	−	−	+	−	+	+

+ indicates acid and clot; −, neither acid nor clot.

Hiss serum water containing 1 per cent of the test substance was used for testing the fermentation properties of these strains. The tubes were incubated at 37°C. for 7 days. Daily observations were made during the period of incubation.

fluid was treated in the cold with the minimal amount of 10 per cent acetic acid necessary to cause complete precipitation; a final concentration of about 0.5 per cent acetic acid was usually required. The precipitate, the so called nucleoprotein fraction, consisting of a mixture of all the bacterial substances precipitated by acetic acid in the cold, was further purified by washing with distilled water or physiological salt solution, redissolving in dilute sodium hydrate solution and reprecipitating with acetic acid. This process was repeated once or twice, the final precipitate washed in water, then very rapidly in acetone and dry ether, after which it was dried *in vacuo*. The resulting fine white powder was stored as the stock material in a vacuum desiccator and subsequently made into solutions by weight as desired. An average yield from 9 liters of original culture was 400 to 500 mg. A few lots were diluted and filtered through Berkefeld V filters at some stage in the preparation, but since considerable loss was experienced by this method and the filtered product gave the same serological results as the unfiltered,

this procedure was abandoned. This antigen has been designated as the nucleoprotein fraction, or for brevity as P. According to Wells (24), such a solution is probably composed of nucleoproteins, nucleins, mucins, "nucleoalbumins," various glycoproteins besides the mucins, simple globulins, and alkaline proteinate formed by the action of alkali on the native protein.

The soluble specific substance was purified from the crude supernatant fluid left after precipitation of the nucleoprotein. With the streptococci it was necessary to recover this substance from the bacterial bodies rather than from the culture fluid, since only a small amount of it is found in solution in streptococcus cultures. The material derived from 18 liters of original broth culture was combined in one lot. The crude solution was evaporated on a steam bath to a small volume (about 50 cc.) and acidified with acetic acid in the cold; and any precipitate was discarded. The clear solution was then boiled in a water bath for 10 minutes in order to remove heat-coagulable proteins, which were also discarded. The supernatant fluid was treated with 10 volumes of 95 per cent alcohol; the resulting precipitate was redissolved in 5 to 10 cc. of water and any insoluble matter discarded after washing with water. The solution, which stood overnight in the ice box between precipitations, was again acidified with dilute acetic acid and again treated with alcohol. This process was repeated about six times, with half the precipitations on the acid and half on the alkaline side. The watery solution was then dialyzed in a parchment bag against running water for 48 hours, or until tests for chlorides, phosphates, and sulfates were negative. In some cases the solution was filtered through a Berkefeld V filter at this stage and concentrated *in vacuo* to about 10 cc.; then one-fourth its volume of 1:1 hydrochloric acid was added in an attempt to precipitate the specific substance, since the Type III pneumococcus specific substance precipitates with this treatment. However, none of the lots so treated with hydrochloric acid showed any precipitate. After standing in the ice box overnight, the hydrochloric acid was removed by dialysis against running water, the solution concentrated to 5 cc. or less, and treated with 10 volumes of acetone. The precipitate was taken up in 1 or 2 cc. of distilled water, reprecipitated with acetone, and dried in a vacuum desiccator to constant weight. The yield from 18 liters of original broth culture was 25 to 30 mg. of flaky yellowish white material. This substance dissolved in physiological salt solution was water-clear and colorless. The solution (referred to for convenience as S) was tested serologically and, to a limited extent, chemically. The presence of further impurities was indicated by a slight residue, insoluble in water, which still remained after the last precipitation.

Easier methods of obtaining the soluble specific substance were sought. In spite of the fact that streptococcus cultures do not autolyze, as pneumococcus cultures do, it was thought that enough S might be secreted by the organism to make the culture filtrate a profitable source of the material. Flasks of broth, inoculated respectively with strains W67, A148, A49, and 38D, were incubated at 37°C.; at intervals of 2 or 3 days during the succeeding month, samples were withdrawn and tested with an antibacterial serum for the presence of a reacting

substance; but only small amounts could be demonstrated at any time. Rapid freezing and thawing of the sedimented bacteria suspended in a small volume of saline gave almost no reacting substance in the supernatant fluid after centrifugation, even though the suspension was frozen and thawed as may as twenty times. Boiling a concentrated suspension of streptococci with various reagents caused the dissociation of S from the bacterial cell. Merely boiling for an hour with physiological saline was effective; although the addition of sodium hydrate or sodium carbonate gave a greater yield. Dissolving the bacteria in antiformin and precipitating out the proteins was also tried successfully. In short, any method of breaking up the cell body is effective in liberating the soluble specific substance. In extraction by boiling with sodium hydrate, the soluble specific substance of certain strains was found to be destroyed, or at least inactivated to a great extent. Consequently, it was felt inadvisable to subject the material to such strong chemical agents as boiling sodium hydroxide or antiformin, and the more tedious method of grinding the dried bacteria and subsequently extracting with N/100 sodium hydroxide in the cold was adhered to throughout.

Solutions of the specific substance prepared as described were subjected to as many qualitative chemical tests as possible with such a small amount of material. In a dilution of 1:400, preparations from the four strains gave negative biuret reactions and positive Molisch tests for carbohydrates. The latter was positive to approximately the limit of serological reactivity of the substance. A positive Molisch test was obtained at a dilution of 1:400,000 with a solution which gave a precipitin test no higher than 1:500,000. Weakly positive reactions for reducing sugars were obtained from Strains W67 and 38D; but after hydrolysis by boiling with concentrated acid strongly positive reactions were obtained with Fehling's solution from all four strains. No precipitate was obtained with phosphotungstic acid in any instance, but with copper sulfate and uranium nitrate a precipitate was formed in the solutions made from Strains W67 and A148 but not with those from A49 and 38D. These solutions gave no color with iodine and were not precipitated with 1:1 hydrochloric acid. The specific substance passed through a Berkefeld V filter and withstood autoclaving and boiling for prolonged periods. Preparations from some strains withstood boiling with N/10 sodium hydroxide for an hour. In dialysis experiments the specific substance was held back by ordinary parchment membranes.

This necessarily meager chemical information at least gives the same indications as the studies of Heidelberger and Avery on pneumo-

coccus. It seems probable that the specific substance of non-hemolytic streptococcus is non-protein in nature. That it may be a polysaccharide is indicated by the positive Molisch test in high dilution together with the presence of reducing sugars after hydrolysis. The differences in precipitation of preparations from different strains by heavy metal salts is interesting in view of similar distinctions between the specific substances from Type II and Type III pneumococci. More detailed chemical analysis must await the collection of large quantities of material.

The serological behavior of the bacterial substances, separated and prepared as noted in the preceding section, was studied by means of sera from rabbits immunized, on the one hand, against the living bacteria and, on the other, against the chemically extracted protein material. Preliminary tests had indicated that the two kinds of antibodies found by Avery and Heidelberger in antipneumococcus sera were also present in sera obtained by immunizing rabbits with living cultures of green streptococci: one antibody for the highly reactive strictly specific soluble substance (S), the other for the less highly reactive relatively non-specific nucleoprotein (P). The antibacterial sera against the four organisms chiefly studied were now tested for antibody content. Twelve antibacterial sera were titrated completely for agglutinins, for S precipitins, and for P precipitins. A number of other rabbit sera, used incidentally, were not titrated completely; but in so far as these were used the results were consistent with those from the sera more thoroughly studied. Two typical protocols of complete titrations are shown (Tables II and V). The records of the other sera are omitted because in their essential features they do not differ from those shown. The one exception will be considered in some detail.

Table II shows the results for the serum of Rabbit L71, immunized with living bacteria of Strain W67. Tested for agglutinins against the four strains in question, it showed a high titer with the homologous organism, at least 1:4,000 serum dilution, and no appreciable cross-agglutination with the other three strains. Similarly precipitation tests with the purified specific substance showed absolute specificity in relatively high dilutions. In this particular serum a good precipitation was obtained only as far as 1:100,000 dilution of S; but

in many sera a titer of 1:500,000 has been obtained, and in a few instances there was undoubted precipitation at 1:1,000,000. This is

TABLE II.

Antibody Content of Antibacterial Serum.

Rabbit L71, immunized for a short period with living culture of Strain W67.

Agglutinin.					Precipitin.								
Serum dilution.	Culture from Strain				Antigen dilution.	S from Strain				Antigen dilution.	P from Strain			
	W67	A148	A49	38D		W67	A148	A49	38D		W67	A148	A49	38D
1:20	++++	−	−	+	1:5,000	++++	−	−	−	1:1,000	+++	±	−	−
1:40	++++	−	−	+	1:10,000	++++	−	−	−	1:2,000	++	±	+	−
1:80	+++	−	−	+	1:50,000	++	−	−	−	1:4,000	++	−	−	−
1:160	+++	−	−	+	1:100,000	+	0	0	0	1:8,000	++	−	−	−
1:1,000	+++	0	0	0	1:500,000	−	0	0	0	1:16,000	+	−	−	−
1:4,000	++	0	0	0						1:32,000	±	−	−	−
1:16,000	+	0	0	0										
1:32,000	±	0	0	0										

++++ indicates complete agglutination or precipitation.

+++, ++, +, ± indicates grades in the series to −, which indicates a negative reaction.

0 indicates that no test was made.

S = soluble specific substance. P = nucleoprotein.

In this table, as in all subsequent ones, the agglutinations were performed by mixing 0.5 cc. of serum dilution (made with broth) with 0.5 cc. of an 18 hour plain broth culture. The tubes were incubated for 2 hours at 56°C. and read immediately.

The precipitations were performed by mixing 0.2 cc. of antigen dilution with 0.1 cc. of serum and 0.1 cc. of physiological salt solution. All dilutions were made with physiological salt solution. The tests were incubated for 2 hours at 37°C. and read after standing 18 hours in the ice box.

In all experiments, complete sets of controls with and without normal serum were included. Unless the controls were negative, the experiment was not considered as evidence. The controls have been omitted from the tables in order to save space.

All dilutions are recorded as final dilutions.

low in comparison with the 1:6,000,000 titers of pneumococcus S observed by Avery and Heidelberger (17), but their figures were obtained with more highly purified material and with serum of horses

subjected to long periods of immunization. In working with rabbits immunized much less intensively and with antigens of no higher degree of purification than those used in this study, it is felt that these figures are comparable to their results.

Much lower titers for P than for S antibodies were obtained in antibacterial sera. The titration of Rabbit L71 serum illustrates this point. Here again significant precipitation was found only with the homologous nucleoprotein, although there was a trace of cross-precipitation with proteins from Strains A148 and A49. The same marked difference has been observed in the character of the precipitate obtained from precipitation of the serum by S and by P as that described by Avery (17) for pneumococcus precipitates. The S precipitate is typically a compact, slightly translucent disk at the bottom of the tube. The flakes which form immediately, after mixture of S and serum settle out very quickly and coalesce to a considerable extent by the end of the 2 hour incubation period. After the tubes have stood overnight in the ice box, it is almost impossible to break up this mass by shaking the tube. This is the condition when precipitation is complete. The incomplete precipitates show varying degrees of compactness and also different amounts of precipitate. This point has been discussed by Morgan (25) for pneumococcus; and Hitchcock (13, 14) has reported the same kind of precipitate in his experience with streptococcus. In contrast to this compact disk, the P precipitate is composed of heavy flocculi which do not coalesce but form a bulky mass of finely divided particles easily shaken up from the bottom of the tube. This difference between S and P precipitates is striking and constant.

The lack of cross-precipitation with nucleoprotein antigens in an antibacterial serum, such as that of Rabbit L71, was a distinct surprise since earlier work using the complement fixation reaction and similarly prepared antigens and sera had clearly indicated consistent cross-fixation of antibacterial sera by heterologous P antigens. Ten rabbits were used in the complement fixation series, with two or three immunized against each organism. Of these, five gave strongly positive cross-fixations, while the other five showed moderate or weak, but still very definite cross-reactions. A protocol of the complement fixations with the serum of one of these rabbits giving strong reactions

is shown in Table III. The proper proportion of antigen and serum for precipitin tests had not been determined at the time this serum was available; but the precipitations indicated only slight reactions with the method used. This is perhaps not surprising in view of the fact that the complement fixation reaction is a more delicate test than precipitin tests.

From the result of the complement fixation reactions it seemed probable that the serum of Rabbit L71 would give some cross-precipitation with P from heterologous strains of *Streptococcus viridans;* but since little cross-precipitation was actually observed, the possibility was suggested that the precipitation with the homologous P antigen was due to traces of S remaining in the nucleoprotein solution and that the serum did not contain any antibodies for P. Accordingly, an attempt was made to remove all traces of S remaining as an impurity in the nucleoprotein antigen derived from W67. The protein was precipitated with acetic acid and carried through a number of additional purifications by washing with saline, redissolving, reprecipitating, and finally dissolving the purified nucleoprotein in its initial volume. All wash waters and supernatant fluids after the various precipitations were tested for S in order to determine the amount of S present as an impurity in the original neucleoprotein solution, as well as to be more certain that the final purified solution did not contain demonstrable quantities of S. The three wash waters were neutralized and tested against serum from Rabbit L71. The original supernatant fluid from the first acetic acid precipitate was not tested in this way. Table IV shows a heavy precipitate with the first wash water, and only slight precipitates with the two succeeding ones. But the wash waters of a nucleoprotein precipitate usually contain varying amounts of P in solution. Consequently, it was thought that probably some of this precipitate was due to P, while some might be due to S. In order to determine how much was due to S, the original supernatant fluid and the wash waters were treated with acetic acid in the cold and then boiled for 10 minutes, still in the acid condition, to remove proteins. After neutralization, the solutions were again tested against the serum of Rabbit L71. Only slight precipitates now appeared. This was interpreted as an indication that most of the precipitate with the first untreated wash water was not attributable

TABLE III.

Complement Fixation Reactions.

Rabbit 49, immunized for a short period with living culture of Strain A148.

Serum.	Antigen. P from Strain			
	W67	A148	A49	38D
cc.				
0.1	+++	++++	++++	++++
0.05	++++	++++	++++	++++
0.025	+++	++++	++++	+++
0.012	+++	++++	++++	+++
0.006	++	++++	+++	+
0.003	++	+++	++	−

In the complement fixation experiments, ½ Wassermann quantities were used. 0.25 cc. of a 1:500 dilution of antigen was used in all tubes. Antigen, guinea pig complement, and immune serum were mixed in a total volume of 0.75 cc. and incubated in a water bath at 37°C. for 1 hour. 5 per cent washed sheep cells were sensitized by mixing with an equal volume of an appropriate dilution of rabbit anti-sheep serum. 0.5 cc. of sensitized cells was added to all tubes of the test, followed by a secondary incubation period of ½ hour. Readings were made after standing overnight in the ice box.

A complete set of controls was included using normal serum, and antigen and serum alone in at least double the largest amounts used in the test. All controls were satisfactory.

TABLE IV.

P from Strain W67 Tested for Traces of S.

Precipitin tests.

	Untreated wash waters.			Wash waters treated with acetic acid cold and boiling.				Purified P.
	1	2	3	Original supernatant fluid.	1	2	3	
Serum of Rabbit L71.....	+++	±	+	−?	±	+	−	++±

The technique described for precipitin tests in Table II was used in this experiment.

to S but to P. The final purified P, presumably free of S, gave as good a precipitate with Rabbit L71 serum as the unpurified control. Nucleoprotein antigens from the other strains gave similar results when tested in the same way against their respective antisera. It seemed apparent, therefore, that the P preparations contained only negligible amounts of S as an impurity, insufficient to account for the specific reactions sometimes obtained with nucleoprotein antigens. Whatever precipitate was present when P was used as antigen in the precipitin test against its homologous antibacterial serum was then chiefly a P precipitate. One must conclude, therefore, that homol-

TABLE V.

Antibody Content of Antibacterial Serum.

Rabbit L92, immunized for a long period with living culture of Strain A148.

Agglutinin.					Precipitin.								
Serum dilution.	Culture from Strain				Antigen dilution.	S from Strain				Antigen dilution.	P from Strain			
	W67	A148	A49	38D		W65	A148	A49	38D		W67	A148	A49	38D
1:40	+	+++	++	−	1:5,000	−	++++	−	−	1:500	+	+++	++	+
1:80	+	+++	++	−	1:10,000	−	++++	−	−	1:1,000	+±	++++	++	+
1:160	−	+++	++	−	1:50,000	−	++++	−	−	1:2,000	+	+++	+	+
1:320	−	+++	++	−	1:100,000	−	++	−	−	1:4,000	+	++	+	+
1:640	−	++	+	−	1:500,000	0	+	0	0	1:8,000	+	++	+	±
1:1,280	−	++	±	−						1:16,000	+	+	+	−
1:2,560	−	±	−	−										

The technique described in Table II was used in this experiment.

ogous nucleoproteins react better than heterologous ones with an antibacterial serum. The reason for this is not entirely clear but this fact seems to indicate individual differences in proteins from different strains, especially in the light of other evidence to be discussed later.

It was, however, possible to obtain sera which showed marked cross-precipitation with heterologous nucleoproteins. The serum of Rabbit L92, immunized over a long period against Strain A148, is an illustration of this point (Table V). Several similar sera have been prepared.

An instance of cross-agglutination was found which corroborated the existing information on the parallelism between agglutination and S precipitation. Immunization with a green streptococcus (V92) isolated from an excised subcutaneous nodule of a rheumatic fever patient in 1923 gave rise to a serum which was precipitated by the soluble specific substance of one of the main strains studied (38D). Five sera against V92 were prepared. Reciprocal cross-precipitation of S was demonstrated; cross-agglutination tests gave the same result. It is evident, therefore, that strains of non-hemolytic streptococci which cross-precipitate the soluble specific substance also cross-agglutinate. The converse had already been shown.

The only exception to these general results of antibody content in the antibacterial sera was encountered in the serum of Rabbit L95, an animal highly immunized by a prolonged course of treatment with Strain A49. As may be seen in Table VI, this serum showed cross-precipitation with S from Strain A148. The corresponding cross-agglutination was slight with the usual technique; but when the incubation period was doubled, or the readings delayed for 18 hours following 2 hours incubation at 56°C., cross-agglutination was more pronounced than with the ordinary method. In Table VI both readings are given.

The anti-P content of this serum should be specially noted. Marked precipitation occurred with P from heterologous strains (Table VI). In fact, better precipitation was observed with P from Strain A148 than with P from the homologous strain. This cross-precipitation of proteins with an antibacterial serum should not be overlooked in evaluating the protein group relationships indicated by study of pure anti-P sera. In antibacterial sera the P antibodies are produced in response to the native protein of the unaltered bacterial cell. Evidence derived from the study of these antibodies should, therefore, be given special weight. Table VI shows that proteins from related Gram-positive cocci react only slightly with this serum, although the same series of antigens gives striking cross-reactions when tested with sera prepared against the chemically extracted proteins.

A careful study of this serum by means of absorption tests with bacteria both from Strain A49 and from Strain A148 failed to throw light on the peculiarities of this exceptional serum. Consequently,

TABLE VI.

Antibody Content of Antibacterial Serum.

Rabbit L95, immunized for a long period with living culture of Strain A49.

Agglutinin.							Precipitin.				
Serum dilution.	Read after 2 hrs. at 56°C.				Read after 2 hrs. at 56°C. and 18 hrs. in the ice box.		Antigen dilution.	S from Strain			
	Culture from Strain				Culture from Strain						
	W67	A148	A49	38D	A148	A49		W67	A148	A49	38D
1:40	−	±	++++	+++	+++	++++	1:5,000	−	++	++++	−
1:80	−	+	++++	±	+++	++++	1:10,000	−	++	++++	−
1:160	−	±	++++	±	++±	++++	1:50,000	−	+	+++	−
1:320	−	+	++++	−	++	++++	1:100,000	0	+	+++	−
1:640	−	±	+++	−	+±	+++	1:500,000	0	+	+	0
1:1,280	−	−	+++	−	+	+++	1:1,000,000	0	0	±	0
1:2,560	−	−	++	−	±	++	1:2,000,000	0	0	−	0
1:5,120	−	−	+	−	0	0					
1:10,240	−	−	±	−	0	0					

Precipitin for P antigen.

Antigen dilution.	*Streptococcus viridans.*				*Streptococcus hæmolyticus.*				Staphylococcus.			Pneumococcus.	
	P from Strain				P from Strain				P from Strain			Antigen. dilution.	Group IV, Pn. IV.
	W67	A148	A49	38D	S3	S60	N.Y.5.	A	R	A	H		
1:133	0	0	0	0	+	+	+	+	++	++	+	1:5,600	+±
1:200	0	0	0	0	0	±	+	±	++	++	+	1:11,200	+
1:500	++	+++	+++	++	±	±	+	−	++	+	+	1:22,400	±
1:1,000	++	++++	++++	++	−	−	−	−	+	−	±	1:44,800	±
1:2,000	++	++++	++++	+	−	−	−	−	±	−	−	1:89,600	±
1:4,000	+	+++	++	+	0	0	0	0	0	0	0		
1:8,000	+	++	++	+	0	0	0	0	0	0	0		
1:16,000	+	++	+	±	0	0	0	0	0	0	0		

The technique described in Table II for precipitin tests was used in this experiment, except in reading part of the agglutination tests as noted in the table.

it is believed that this is probably an example of a single animal giving an unusual response to immunization. Table VII shows the result of an absorption experiment performed with the serum of this rabbit

(L95). Complete removal of agglutinins by repeated absorptions with the homologous organism (A49) resulted in the removal from the serum of all antibodies including those for the heterologous S from Strain A148 which cross-precipitated with this serum. Absorption

TABLE VII.

Absorption Experiment.

Rabbit L95, immunized for a long period with living culture of Strain A49.

Lot.	Rabbit L95 serum.	Agglutination.							Precipitation.		
		Tested against Culture A49.							Antigen dilution.		
									Strain A49.		Strain A148.
		Serum dilution.							S	P	S
	Absorbed with	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:10,000	1:1,000	1:12,500
1	Bacteria, Strain A49 (homologous organism).	−	−	−	−	−	−	−	−	−	−
2	Bacteria, Strain A148 (heterologous organism).	+++	++	++	++	+	+	+	++?	−	−
3	Untreated controls Rabbit L95 serum.	++++	++	++	++	+	+	+	++	++	+
4	Normal rabbit serum.	−	−	−	−	−	−	−	−	−	−

This experiment was performed by absorbing the serum in a dilution of 1:4 with the bacterial sediment from the strain indicated for 2 hour periods at 37°C. Nine repetitions were required in order to remove all agglutinins from Lot 1. All other lots were given the same amount of heating and dilution. The sediment from 500 cc. lots of plain broth culture, heated 1 hour at 56°C., was used for the first seven absorptions. The sediment from 3 liter lots was used for the last two absorptions.

under the same conditions with bacteria from the heterologous strain did not affect the agglutinins nor the S precipitins for the strain used in immunization but removed the S antibodies for Strain A148 and all P antibodies as well. The latter fact tends to confirm the belief that the nucleoproteins of the non-hemolytic streptococci are essen-

tially alike in their serological behavior. The absorption of S antibodies for Strain A148 by bacteria from Strain A49 cannot be considered indicative of subgroup relationships since the sera of none of the four other rabbits immunized with Strain A49 showed any cross-precipitation with S from Strain A148. As stated above, it is felt that this rabbit must be one which has given an unusual response to immunization, due to the peculiarities of the individual animal.

An attempt was made to correlate the serological relationship between S and P antibodies and agglutinins in antibacterial sera by means of absorption experiments. An experiment was performed with the serum of Rabbit L71, immunized with living bacteria of Strain W67. Table VIII, Part 1, shows the results after several absorptions with S and P. After four absorptions the lot treated with the homologous S failed to give any further S precipitate, but still precipitated with P as strongly as the control. This serum still gave good agglutination of the homologous strain. It is uncertain whether this agglutination was due to the P antibodies, demonstrable in the serum at that time, or to traces of S antibody no longer detectable by the precipitin test. Lot 2 of the serum after complete absorption of P antibodies showed undiminished reactivity with S, and agglutination of about the same strength as the untreated control. Table VIII, Part 2, gives the final result of this experiment. Lot 1 after complete absorption with S was further treated with P to remove the P antibodies from the serum. Similarly, Lot 2 after complete removal of P antibodies was treated with S to remove the S antibodies, a procedure which removed the agglutinins in both cases.

This experiment brought out several important points:

1. Absorption with S did not remove P antibodies.
2. Absorption with P did not remove S antibodies.
3. The S antibody was closely related to the production of agglutination.

Detailed daily tests in this and in other similar experiments showed that small traces of S antibody are probably sufficient to produce almost maximal agglutination, while P antibodies are little, if at all, effective in causing agglutination. These facts are construed to mean that the antibodies responsible for S precipitation are probably also operative in agglutination.

TABLE VIII.

Absorption Experiment.

Rabbit L71, immunized for a short period with living culture of Strain W67.

Lot.	Rabbit L71 serum.	Agglutination. Tested against Culture W67. Serum dilution.					Precipitation. Antigen dilutions.	
							S	P
	Absorbed with homologous	1:40	1:80	1:160	1:320	1:640	1:5,000	1:400
Part 1. Incomplete absorption.								
1	S	++	++	++	++	+	−	++±
2	P	++	++	+++	++++	++++	++++	−
3	Untreated controls. Rabbit L71 serum.	++	+++	++++	++++	++++	++++	++±
4	Normal rabbit serum.	−	−	−	−	−	−	−

							S	P
Part 2. Complete absorption.							1:5,000	1:200
1	S to completion, then P.	±	±	−	−	−	−	−
2	P to completion, then S.	+	+	+	±	−	−	−
3	Untreated controls. Rabbit L71 serum.	++++	++++	++++	++++	++++	++++	++±
4	Normal rabbit serum.	−	−	−	−	−	−	−

Serum was diluted 1:2 and appropriate amounts of antigen added to make the final antigen concentration the optimal for precipitation. The tubes were incubated for 2 hours at 37°C. and left in the ice box overnight. After the precipitate had been removed by centrifugation, more antigen was added and the procedure repeated until no further precipitate formed. The sera were then tested for agglutinins and for S and P precipitins as indicated in Part 1 of the table. The remaining serum in Lots 1 and 2 was further absorbed with P or with S, as indicated in Part 2 of the table, until absorption was complete. These sera were again tested for agglutinins and for S and P precipitins as recorded in Part 2 of the table.

In conducting the absorption experiments, it was apparent from the first that the procedure was difficult and had many possibilities of error. While not many such experiments have been performed, it is felt, nevertheless, that they are of value in that they confirm the data obtained by other serological reactions.

SUMMARY.

1. Agglutination and precipitation by the specific substance of *Streptococcus viridans* are parallel phenomena. Separate specific substances have been extracted from strains which are distinct by ordinary serological tests. Preliminary chemical examination indicates that the specific substances may be complex carbohydrates.

2. A close relationship between nucleoproteins from different strains of *Streptococcus viridans* is suggested by the existence of a certain amount of cross-precipitation and a larger degree of cross-complement fixation. But the occurrence of stronger reactions with homologous nucleoproteins than with heterologous indicates that there is some degree of individual difference in proteins from separate strains.

3. Two distinct antibodies are present in the sera antibacterial for *Streptococcus viridans:* one of high titer implicated in the parallel phenomena of agglutination and precipitation by the soluble specific substance, the other usually of low titer and involved in precipitation by nucleoproteins but probably little, if at all, in agglutination.

The significance of these results obtained from the study of antibacterial sera will be considered in the general discussion of the antigenic components of *Streptococcus viridans* after the results from the study of antinucleoprotein sera have been presented in the succeeding paper.

BIBLIOGRAPHY.

1. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73.
2. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 81.
3. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1924, xl, 301.
4. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.
5. Fornet, W., *Münch. med. Woch.*, 1906, liii, 1862.
6. Ascoli, M., cited by Kraus, R., in Kolle, W., and Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 750.
7. Hamburger, cited by Kraus, R., in Kolle, W., and Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 750.

8. Zinsser, H., *J. Exp. Med.*, 1921, xxxiv, 495.
9. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275.
10. Mueller, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 209.
11. Mueller, J. H., and Tomcsik, J., *J. Exp. Med.*, 1924, xl, 343.
12. Petroff, S. A., *Am. Rev. Tuberc.*, 1923, vii, 412.
13. Hitchcock, C. H., *J. Exp. Med.*, 1924, xl, 575.
14. Hitchcock, C. H., *J. Exp. Med.*, 1924, xl, 445.
15. Przesmycki, F., *J. Infect. Dis.*, 1924, xxxv, 537.
16. Landsteiner, K., van der Scheer, J., and Witt, D. H., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 289.
17. Avery, O. T., and Heidelberger, M., personal communication.
18. Kinsella, R. A., and Swift, H. F., *J. Exp. Med.*, 1917, xxv, 877.
19. Kinsella, R. A., and Swift, H. F., *J. Exp. Med.*, 1918, xxviii, 169.
20. Hitchcock, C. H., *J. Exp. Med.*, 1925, xli, 13.
21. Meyer, K. F., and Christiansen, C. R., *J. Infect. Dis.*, 1917, xx, 391.
22. Eberson, F., *J. Immunol.*, 1920, v, 345.
23. Woolridge, and Du Bois-Reymond, cited by Přibram, E., in Kolle, W., and Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 1361.
24. Wells, H. G., The chemical aspects of immunity, 1925, Chemical Catalog Company, New York, 35.
25. Morgan, H. J., *J. Immunol.*, 1923, viii, 449.

THE IMMUNOLOGICAL RELATIONSHIPS OF STREPTOCOCCUS VIRIDANS AND CERTAIN OF ITS CHEMICAL FRACTIONS.

II. Serological Reactions Obtained with Antinucleoprotein Sera.

By REBECCA C. LANCEFIELD.

(From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, and the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, April 22, 1925.)

In the preceding paper (1) a study of the reactive and antigenic constituents of *Streptococcus viridans* was made by an analysis of the antibody content of antibacterial sera. It was shown that such sera contain relatively non-specific as well as specific antibodies. The specific antibody, apparently a single one, is probably concerned with the parallel phenomena of agglutination and precipitation of the serum by the specific carbohydrate chemically extracted from the cell. The non-specific antibody shows group reactions with nucleoproteins of serologically distinct strains.

In order to investigate the group relationships of the nucleoproteins, it seemed advisable to immunize rabbits to this fraction of the bacterial cell. The preparation of this material has already been described (1). The method of immunization was to give repeated series of daily intravenous injections of small doses for 4 or 5 days, followed by a rest of 3 days. Usually four to six series were required to produce antisera of suitable titer. These sera deteriorate on standing in the ice box for a few months. Table I shows the results obtained in titrating four such sera against protein antigens. The chief points of interest were as follows:

1. Cross-precipitation was universal. The homologous antigen was usually slightly more effective than heterologous ones, but the difference was not marked and was probably due to the peculiarities of individual antigens resulting from unavoidable denaturization in

TABLE I.

Precipitins in Sera of Rabbits Immunized with P.

Antigen dilution.	Rabbit L59. Immunized with P from Strain W67.					Rabbit L63. Immunized with P from Strain A148.				
	Antigen for precipitin test. P from Strain					Antigen for precipitin test. P from Strain				
	W67	A148	A49	38D	*S. hæmolyticus.*	W67	A148	A49	38D	*S. hæmolyticus.*
1:200	0	0	0	0	0	−	−?	−?	±	−
1:500	0	0	0	0	0	−	−?	−?	−	−
1:1,000	0	0	0	0	0	−?	±	±	−	−
1:2,000	0	0	0	0	0	±	+	+	−	+?
1:5,000	++++	+++	+++?	+?	++±	+±	0	+±	±	+?
1:10,000	+++	++±	+++?	+?	++	+±	+±	+	−	+?
1:20,000	++±	+	++	+?	+	±	+	±	−	−
1:40,000	±	±	±	−	±	−	±	−?	−	−
1:80,000	−	−	−	−	−					

Antigen dilution.	Rabbit L57. Immunized with P from Strain A49.					Rabbit L65. Immunized with P from Strain 38D.				
	Antigen for precipitin test. P from Strain					Antigen for precipitin test. P from Strain				
	W67	A148	A49	38D	*S. hæmolyticus.*	W67	A148	A49	38D	*S. hæmolyticus.*
1:200	0	0	0	0	0	−	−	−	+?	−
1:500	0	0	0	0	0	−	−	−	+	±
1:1,000	0	0	0	0	0	±	±?	±	++	+
1:2,000	0	0	0	0	0	+	±	+	++±	++
1:5,000	++	+++	+++	±	++	++	+?	+±	++±	++±
1:10,000	++	++	++±	+?	+±	+±	+?	+	+±	+±
1:20,000	+	+	++	−	+?	+	±	+?	±	+
1:40,000	±	±	−?	−	±	−?	−	±	−	+

All strains in this table except *Streptococcus hæmolyticus* are *Streptococcus viridans.*

The precipitations were performed by mixing 0.2 cc. of antigen dilution with 0.1 cc. of serum and 0.1 cc. of physiological salt solution. All dilutions were made with physiological salt solution. The tests were incubated for 2 hours at 37°C. and read after standing 18 hours in the ice box. In all experiments complete sets of controls with and without normal serum were included. Unless the controls were negative, the experiment was not considered as evidence. The controls have been omitted from the tables in order to save space.

All dilutions are recorded as final dilutions.

TABLE II.

Precipitin Titration.

P antibodies in serum of Rabbit L90.
Immunized with P from *Streptococcus viridans* A49.

Antigen: P from	Antigen dilution. 1:1,000	1:2,000	1:4,000	1:8,000	1:16,000	1:32,000
Streptococcus viridans A49.	++++	++++	++++	++++	+++	++
" " AW67.	+++	+++	+++	+++±	+++	++
" " A148.	+++	++++	++++	+++	++	++
" " 38D.	++	+++	++++	++++	+++	++
" *hæmolyticus* S3.	+++	++++	++++	+++	+++	++
" " S60.	+++	+++	+++	+++	++	++
" " N. Y. 5.	+++	+++	+++	++++	+++	++
" " A.	+++	++++	++++	++++	++	++
Staphylococcus R.	+	++	++	++	+	+
" A.	+	++	++	+	+	+
" H.	±	+	+	+	+	+
B. coli.	−	−	−	−	−	−
" *diphtheriæ.*	−	−	−	−	−	−

	Antigen dilution.* 1:5,600	1:11,200	1:22,400	1:44,800	1:89,600	1:179,200	1:358,400	1:616,200	1:1,433,600
Pneumococcus Group IV.	+	++	++++	++++	++±	+±	+	±	−

* Dilutions of this antigen were based on determinations of the nitrogen content of the solution. All other antigen dilutions were based on solutions made up by weight from the dried material.

The technique described for precipitin tests in Table I was used in this experiment.

TABLE III.

Absorption Experiment.

P antibodies in serum of Rabbit L90.
Immunized with P from *Streptococcus viridans* A49.

Antigen in precipitin test. Dilution 1:5,000. P from Strain	Controls.		P antibodies in serum of Rabbit L90 after absorption with P from												
	Unabsorbed serum of Rabbit L90.	Normal rabbit serum.	*Streptococcus viridans* Strain				Pneumococcus Group IV, Pn. IV.	*Streptococcus hæmolyticus* Strain				Staphylococcus Strain		
			A49	W67	A148	38D		S3	S60	N.Y.5.	A	R	A	H
Streptococcus viridans A49.	++++	−	−	−?	−	±?	−?	+	+	+	+±	++±	+++	++++
" " W67.	++++	−	−	−?	0	0	−?	−	±	0	−?	++	0	0
" " A148.	++++	−	−	±	−	0	±	+	++±	+	+	+++±	0	++++
" " 38D.	++++	−	−	−	0	−	−?	−?	+	0	−?	++±	0	0
Pneumococcus Group IV, Pn. IV. (dilution 1:44,800).	++++	−	−	±	0	0	−?	0	++	0	0	++	0	0
Streptococcus hæmolyticus S3.	+++	−	−	−	0	0	−	−	−	0	0	++	0	0
" " S60.	+++	−	−	−	0	0	−	−	−	0	0	++	0	0
" " N. Y. 5.	++++	−	−	−	0	−	−	−	−	±?	0	++±	+++	++±
" " A.	++++	−	−	−	−	0	−	−	−	−	−	++	0	0
Staphylococcus R.	++	−	−	−	−	0	−	−	−	0	0	−	−	0
" A.	++	−	−	−	−	−	−	−	−	−	0	−	−	−
" H.	++	−	0	0	0	−	0	−	−	−	0	−	−	−
Saline control.	−	−	−	−	−	−	−	−	−	−	−	−	−	−

The experiments recorded in this table and Table V were performed in the following way. Serum was diluted 1:3 and antigen added to make a final concentration of 1:4,000. The precipitin titrations had shown that these were the optimal dilutions for precipitation. The tubes were incubated for 2 hours at 37°C. and then placed in the ice box overnight. After removal of the precipitate by centrifugation, antigen was again added to a final concentration of 1:4,000. The tubes were again incubated for 2 hours at 37°C. and left overnight in the ice box. This procedure was repeated until no further precipitate was formed. Usually three or four absorptions were sufficient to remove all antibodies for the antigen used as absorbent. Precipitin tests were made with 0.3 cc. of the absorbed serum (the equivalent of 0.1 cc. of original undiluted serum) added to 0.1 cc. of suitable antigen dilutions. Several antigens were titrated in final dilutions of 1:2,000, 1:4,000, and 1:8,000 against representative lots of absorbed serum. Only one dilution is recorded for each test since consistent results were obtained in all tubes. Readings were made after 2 hours at 37°C. and overnight in the ice box.

preparation. Thus antigen P from Strain 38D was suspected of having undergone considerable denaturization; the results in Table I show that it precipitated heterologous sera only slightly, and that its antiserum was only slightly precipitated by other antigens.

2. Prozone phenomena were observed. At an antigen dilution of 1:200 or even 1:1,000 no precipitation occurred in the serum of Rabbits L63 and L65, while at a dilution of 1:5,000 good precipitations were found throughout the series. Similar prozones occurred in all antiprotein sera.

3. In a given serum, the titration curves were almost identical for the various antigens. A protein antigen from hemolytic streptococcus showed the same curves as the non-hemolytic antigens.

Complement fixation tests with the same series of antigens and sera confirmed the findings of Table I. In addition it was found that none of these sera was precipitated by S (the soluble specific substance) from any source.

An absorption experiment was performed with the best sera of this series (Rabbit L59 serum), using as antigens the protein solutions prepared from four different strains of non-hemolytic streptococcus. After six successive treatments of a sample of this serum with its homologous protein solution, no further precipitate was obtained on addition of the homologous protein or of any heterologous green streptococcus protein. Proteins from three different strains were used in similar experiments to absorb samples of the same anti-P serum (Rabbit L59 serum) with the result that each heterologous protein removed all P antibodies from the serum. These absorption experiments confirmed the indications of the precipitin and complement fixation tests that the nucleoproteins of non-hemolytic streptococci are serologically similar.

With the cooperation of Dr. Avery, who furnished pneumococcus protein solutions, proteins from Types I, II, and III pneumococcus and from a Group IV strain were used as antigens in precipitin tests against the four green streptococcus anti-P sera then available. Each protein solution precipitated all four sera, an indication of group relationship between these species. More information was obtained concerning the relationship of pneumococcus and streptococcus proteins by absorption experiments with another antiprotein serum.

Rabbit L90 was immunized with the original unpurified N/100 sodium hydroxide extract of a non-hemolytic streptococcus. Tables II and III show the result of testing this serum by means of precipitin reactions and absorption tests with protein antigens from four non-hemolytic streptococci, from four strains of hemolytic streptococci, from three different staphylococci, and finally from a strain of *Bacillus coli* and from one of *Bacillus diphtheriæ* as controls. The antiserum against the green streptococcus protein was also tested with pneumococcus protein. The hemolytic streptococcus strains were from the following sources: one, scarlet fever, N.Y.5, obtained from Dr. A. R. Dochez, one erysipelas, and the other two were representatives of two of the groups studied by Dochez, Avery, and Lancefield in 1919 (2). Another anti-P serum prepared against protein from a hemolytic streptococcus, was tested with all these antigens except the pneumococcus protein. The results are shown in Tables IV and V. From the precipitin tests alone, it seemed that the hemolytic and the non-hemolytic streptococcus proteins were interchangeable; both antisera were precipitated equally well by proteins from either group. Pneumococcus protein precipitated the serum of Rabbit L90 (Table II) better than any other antigen in the series. Protein from staphylococcus, on the contrary, was consistently less reactive than either of the streptococcus antigens, while the proteins from unrelated groups represented by strains of *Bacillus coli* and *Bacillus diphtheriæ*, failed to precipitate these sera in any dilution. It is noteworthy that in this series all proteins giving positive results of any sort precipitated the serum to approximately the same dilution of antigen. In spite of the fact that the recorded antigen dilutions are not entirely accurate, since they are based on weights of dried nucleoproteins rather than on chemical determinations of protein nitrogen present in the solutions, the difference in the several antigens seemed to be represented not by the range of antigen dilution over which precipitation occurred but rather by the amount of precipitate produced.

Absorption tests made with each serum and the twelve antigens of the four groups detailed above, gave more exact information concerning the interrelationships than was afforded by precipitin tests. The results are recorded in Tables III and V. By these experiments it was shown that absorption with protein from any organism of the

group used in immunization removed all antibodies from the serum. For example, in absorption tests with the serum of Rabbit P976, immunized against protein from a hemolytic streptococcus, every hemolytic streptococcus protein absorbed all antibodies for hemolytic and non-hemolytic streptococcus proteins and for staphylococcus

TABLE IV.

Precipitin Titration.

P antibodies in serum of Rabbit P976.
Immunized with P from *Streptococcus hæmolyticus* S3.

Antigen: P from	Antigen dilution.					
	1:1,000	1:2,000	1:4,000	1:8,000	1:16,000	1:32,000
Streptococcus hæmolyticus S3.	++?	++	++	++?	+	+
" " S60.	++	++	+++	++	+	±
" " N. Y. 5.	++	++	+++±	+++±	++	+
" " A.	+++	+++	+++±	+++±	++	++
" *viridans* A49.	+	+±	++	++	++	+±
" " W67.	±	±	++	+±	+±	+±
" " A148.	+	+±	++	++	+±	+
" " 38D.	±	+	++	++	++	+±
Staphylococcus R.	+	±	+	+	+	±
" A.	±	±	+±	+±	+	+
" H.	±	±	+	±	±	±
B. coli.	−	−	−	−	−	−
" *diphtheriæ.*	−	−	−	−	−	−

The technique described for precipitin tests in Table I was used in this experiment.

proteins as well. A green streptococcus protein, however, absorbed all antibodies for green streptococcus and for staphylococcus proteins but only reduced the titer for hemolytic streptococcus protein without completely removing the antibodies for the latter. A much smaller proportion of the total antibody was absorbed by staphylococcus protein; it removed all staphylococcus antibodies and reduced the titer slightly for proteins of the other groups. A diagram (Fig. 1)

TABLE V.

Absorption Experiment.

P antibodies in serum of Rabbit P976.
Immunized with P from *Streptococcus hæmolyticus* S3.

Antigen in precipitin test. Dilution 1:4,000. P from Strain	Controls.		P antibodies in serum of Rabbit P976 after absorption with P from										
			Streptococcus hæmolyticus Strain				*Streptococcus viridans* Strain				Staphylococcus Strain		
	Unabsorbed serum of Rabbit P976.	Normal rabbit serum.	S3	S60	N.Y.5.	A	A49	W67	A148	38D	R	A	H
Streptococcus hæmolyticus S3.	++	−	−	−	−	−	+	+	+	±	++	+±	++
" " S60.	++±	−	0	−	−	−	0	+	+	+	0	+±	++
" " N. Y. 5.	+++	−	−	−	−	−	0	+	+	+	0	++	++
" " A.	+++	−	0	−	−	−	0	+	+	+	0	+±	+±
" *viridans* A49.	++±	−	−	−	−	−	−	−	−	−	+	+	+
" " W67.	+±	−	0	−	−	−	0	−	−	−	0	+	+
" " A148.	++	−	0	−	−	−	0	−	−	−	0	+	+
" " 38D.	++	−	0	−	−	−	−	−	−	−	0	+	+±
Staphylococcus R.	+	−	−	−	−	−	−	−	−	−	−	−	−
" A.	++	−	0	−	−	−	−	−	−	−	−	−	−
" H.	+	−	0	−	−	−	−	−	−	±	0	−	−
Saline control.	−	−	−	−	−	−	−	−	−	−	−	−	−

The technique described in Table III was used in this experiment.

makes this relationship clearer. Staphylococcus protein contains some fraction, represented in the diagram as A, in common with the two kinds of streptococci. This common fraction must be a small part of the whole complex, as shown by the slight precipitate and by the slight reduction in titer for streptococcus antigens after absorption of the serum with staphylococcus protein. The hemolytic and non-hemolytic streptococci have some rather large fraction B in common as indicated by heavy cross-precipitation and the large amount of cross-absorption. However, a certain residue remains which is distinctive for each kind of nucleoprotein. The distinctive parts of the

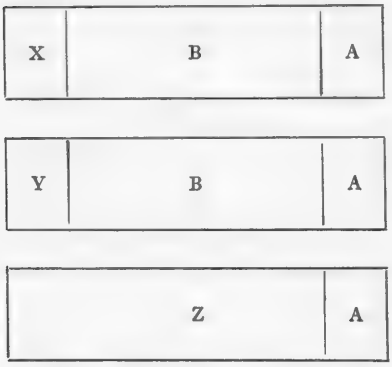


FIG. 1. Diagram to illustrate results of absorption experiments with anti-protein sera. No attempt has been made to diagram the relationship of pneumococcus protein to the proteins represented above since insufficient data are available for this purpose.

proteins are represented diagrammatically as X, Y, and Z for proteins from the different organisms. The structure of the diagram is probably the same for all members of a given group indicating that the proteins of any one group are serologically similar. No diagram has been made to show the relationship of pneumococcus protein to proteins from the other Gram-positive cocci, since insufficient data are available for more than a general statement of its immunological relationship. Tables II and III show that pneumococcus protein has fractions in common with the other proteins studied, in that cross-precipitation and cross-absorption were observed in the experiment with the serum from Rabbit L90. Pneumococcus protein seemed

more closely related to green streptococcus protein than to the protein of any other group and in addition the pneumococcus and streptococcus proteins showed closer relationship, one with the other, than was found between the proteins of any other groups studied.

It has been found impossible to produce an antiserum for staphylococcus protein of sufficient strength to carry out an absorption experiment. The cross-precipitations, using a weak staphylococcus serum against the series of proteins tested previously, gave indications that crossing occurs, but the antibody content of the serum was too slight to permit satisfactory absorption tests.

From these indications of the group relationships of the proteins of the Gram-positive cocci, it seems reasonable to suppose that the extensive group crossing among the hemolytic and the non-hemolytic streptococci demonstrated by Kinsella and Swift (3, 4) is referable to the delicacy of the complement fixation test in detecting protein antigens and antibodies. Hitchcock's (5) demonstration, with the same test, of group relationships between the streptococci and the pneumococci is explainable on the same basis as due to the presence of similar protein fractions in these closely related species.

Non-Antigenic Nature of S.

The antinucleoprotein sera furnished indirect evidence concerning the production of antibodies by injection of S. The nucleoprotein antigens used in the preparation of antisera all contained demonstrable quantities of S, not completely removed in the course of purification. The six rabbits immunized with nucleoprotein received between twenty-five and thirty intravenous injections over periods of 6 to 8 weeks. No S antibodies appeared in their sera. In order to determine whether S can function as an antigen after dissolution of the cell complex, Rabbit L90 was immunized with the original unpurified N/100 sodium hydroxide extract of pulverized bacteria. At no time during immunization could S antibodies be demonstrated in the serum of this animal, although the material injected contained in solution all the S obtainable by this method from the bacterial sediment of 7 liters of original broth culture. This is at least indirect evidence that S from non-hemolytic streptococci does not, after dissociation from the cell, lead to antibody production. On account of the limited

supply of purified material no direct experiments have been performed yet in the attempt to immunize with chemically isolated S. However, the evidence from the antiprotein sera gives the same indications as the results of others who have tried to immunize with S. Avery, (6) after repeated and extensive efforts, has reported complete lack of success in immunizing with this material from pneumococcus; Zinsser and his associates (7–10), failed to produce antibodies to their bacterial "residue antigens;" Mueller and Tomcsik (11) did not succeed in immunizing rabbits with their purified specific carbohydrate from yeast; in fact, no one has thus far been able to produce S antibodies except by injection of the intact cell body.

DISCUSSION.

The significance of the results obtained with both antibacterial and antinucleoprotein sera are considered in this discussion and summary.

A study of the different kinds of antigens and their corresponding antibodies has shown an antigenic complex for *Streptococcus viridans* entirely analogous to that found in pneumococcus by Avery and Heidelberger (12, 13). Certain differences occur, due perhaps in some measure to differences in "cell architecture" (6), but in its main outlines the situation is the same. Under appropriate conditions, two different antibodies may be produced: one an antibody for the bacterial protein which shows group reactions with proteins from related species, and the other an antibody for the non-protein, probably carbohydrate substance. This soluble specific substance is only antigenic when in the unaltered cell complex. The antibody for the latter is precipitated by the specific soluble substance chemically extracted from the cell and is closely related to specific agglutination of the homologous bacteria. Specific agglutination and S precipitation are parallel. Table VI summarizes the antibody response to the two kinds of antigens. Living bacteria stimulate in the rabbit specific agglutinins and S antibodies, which are probably identical according to absorption experiments; but intact bacteria may also lead to the production of non-specific antibodies whose presence can be demonstrated by precipitation and complement fixation with the protein fractions of homologous and heterologous strains. It is possible that P antibodies in such a serum may be responsible for slight non-specific cross-agglutination.

The study of the antigenic complex of the non-hemolytic streptococci has been extended to include the relationships between the proteins of the common pathogenic Gram-positive cocci. The sera prepared for this purpose against cell extracts largely composed of protein (although some S is always present as an impurity) give rise solely to protein antibodies which may be demonstrated either by the precipitin or the complement fixation reaction. Cross-precipitation and absorption experiments with these sera have been used to show certain group relationships among the proteins of related Gram-positive cocci. That cross-precipitation is not a common characteristic of nucleoproteins from all bacterial species is shown by the lack of cross-reactions with proteins prepared from organisms of unrelated groups. It is not yet known whether the serological relationships of

TABLE VI.

Antigenic Value of Cell Constituents of Streptococcus viridans.

Rabbit.	Serum tested for					
	Agglutinin.		Precipitin.			
			S		P	
Immunized with	Homol-ogous.	Heterol-ogous.	Homol-ogous.	Heterol-ogous.	Homol-ogous.	Heterol-ogous.
Living bacteria.	++++	±?	++++	−	++	+
P (containing S as an impurity).	±	−	−	−	++++	++++

similar protein fractions in related species are dependent upon separate chemical compounds or upon certain radicals possessed in common. There is a possibility that the cross-reactions are entirely due to denaturization of the native protein by chemical manipulation. Even if this is true there must be some fundamental similarity in the original proteins which leads to similar products of denaturization. In this connection it should be recalled that certain antibacterial sera with a high content of P antibodies show indiscriminate cross-precipitation within the group but only slight reactions when tested against proteins from related bacterial species. In antibacterial sera, in which the untreated intact bacterium is used in immunization, the response must be reflected in antibodies more nearly directed against the native

bacterial protein than is possible in sera prepared by immunization with the chemically treated protein extracts which are subject to denaturization in the process of preparation. It is hard to interpret the facts relating to cross-relationships among the proteins, since the results obtained by use of the antibacterial sera do not altogether confirm the results shown by sera prepared against the chemically extracted proteins. The majority of the evidence, however, seems to indicate group relationships based on immunological similarities in the bacterial proteins.

Only a small amount of homologous agglutinins have been found in anti-P sera. Potent sera of this kind usually agglutinate the homologous strain in the lower dilutions, but give no agglutination with heterologous strains. The failure of anti-P sera to agglutinate heterologous strains may be correlated with the better precipitation of antibacterial sera by proteins from homologous strains than from heterologous. However, in view of the evidence from cross-precipitation and absorption experiments with both kinds of antisera, one must conclude that although serologically the bacterial proteins of different strains of green streptococci are not necessarily identical, they are nevertheless closely related.

The structure of the cell probably plays an important rôle in determining the amount of agglutination that may be caused by P antibodies. Thus it has been found (6) that in ordinary pneumococcus cultures no real anti-P agglutination occurs, but rather a precipitation caused by the ready autolysis of pneumococcus suspensions and the consequent liberation of protein into solution. However, pneumococci which by adventitious circumstances have been deprived of all S-producing function and simultaneously of the surrounding capsule, are agglutinable by P antibodies. In streptococcus, the distribution of S material is probably different from that in pneumococcus. In the latter, the S substance is chiefly present in the capsule while the streptococcus has no such ectoplasmic zone. Attempts to obtain the S substance from streptococci indicate that this material is distributed through the cell body, or at least is in the interior, since rupture of the cell itself is necessary in order to obtain the S substance in any quantity. With such a distribution of cellular materials, it is conceivable that in the streptococcus some of the protein constituents are

at the surface of the cell and render it, therefore, subject to the action of P antibodies and subsequent agglutination.

No S antibodies have been found in anti-P sera, although in one instance the whole cell extract was used for immunization without any removal of S. No direct attempts to produce S antibodies have been made for streptococcus, but the results with the anti-P sera are indicative of the probable futility of such attempts with the purified material, especially in view of the failure of all other workers to produce antibodies with purified specific substances.

Certain attempts have been made to determine the chemical nature of the specific soluble substance. Only qualitative tests could be made with the limited supply available, but the preliminary chemical findings are submitted. This substance is not only serologically entirely analogous in its behavior to the pneumococcus soluble specific substance, but chemical examination has shown that it is non-protein by the ordinary tests and that it contains carbohydrates which give reducing sugars on hydrolysis. Such a substance is strikingly suggestive of the polysaccharides described by Heidelberger and Avery (13, 14). All the evidence so far accumulated indicates the possibility of a similar chemical composition for the soluble substance of the non-hemolytic streptococcus.

SUMMARY.

1. The immunological behavior of two cell constituents of non-hemolytic streptococci has been studied. (*a*) One, the so called nucleoprotein, is relatively non-specific and gives rise to an antibody which shows group reactions with nucleoproteins of related species. (*b*) The other is non-protein by qualitative chemical tests. Preliminary chemical examination has indicated that it may be a carbohydrate. Although this substance is highly reactive with the specific antibodies produced by the intact bacterial cell, yet in its chemically purified condition it is probably non-antigenic. Specific serological reactions with this substance are closely related to specific agglutination of the microorganism.

2. The study of sera prepared by immunization with the chemically extracted protein has shown the presence of antibodies for nucleoproteins alone. No antibodies against the specific soluble substance

have been found in these sera. The protein antibodies are little, if at all, concerned in causing agglutination. Precipitin tests, complement fixation reactions, and absorption experiments have been used to analyze the group relationships with the nucleoproteins of other species. The proteins of each species of Gram-positive cocci studied were immunologically similar within the species and showed definite relationships to the proteins from related species. Proteins from bacteria of unrelated species did not react with antisera against streptococcus protein.

3. Two distinct antibodies have been demonstrated in antisera prepared against living bacteria. By prolonged immunization it was found possible to produce sera with a high content of protein as well as specific antibodies. With ordinary methods, however, the immune sera had a low content of relatively non-specific protein antibodies but a high titer for specific antibodies. The specific antibodies were not reactive with proteins but were active with high dilutions of the soluble specific substance and were responsible for the parallel specific agglutination. Absorption experiments showed that the two antibodies in antibacterial sera were immunologically distinct.

BIBLIOGRAPHY.

1. Lancefield, R. C., *J. Exp. Med.*, 1925, xlii, 377.
2. Dochez, A. R., Avery, O. T., and Lancefield, R. C., *J. Exp. Med.*, 1919, xxx, 179.
3. Kinsella, R. A., and Swift, H. F., *J. Exp. Med.*, 1917, xxv, 877.
4. Kinsella, R. A., and Swift, H. F., *J. Exp. Med.*, 1918, xxviii, 169.
5. Hitchcock, C. H., *J. Exp. Med.*, 1925, xli, 13.
6. Avery, O. T., personal communication.
7. Zinsser, H., *J. Exp. Med.*, 1921, xxxiv, 495.
8. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275.
9. Mueller, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 209.
10. Petroff, S. A., *Am. Rev. Tuberc.*, 1923, vii, 412.
11. Mueller, J. H., and Tomcsik, J., *J. Exp. Med.*, 1924, xl, 343.
12. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 81.
13. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73.
14. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1924, xl, 301.

COMPENSATORY HYPERTROPHY OF THE THYMUS GLAND IN THE RAT.*

By J. MARMORSTON-GOTTESMAN, M.D., and HENRY L. JAFFE, M.D.

(*From the Division of Laboratories of Montefiore Hospital and the Hospital for Joint Diseases, New York.*)

(Received for publication, June 3, 1925.)

We recently have reported several studies concerning the marked stimulation of growth and regeneration that occurs in the thymus gland of rats, of any age, following double suprarenalectomy (1–3). We showed that, as the result of such a procedure, marked hyperplasia of the involuting thymus, with enlargement of the same, occurs with great constancy.

In line with these studies, we undertook a series of transplantation experiments which involved the carrying out of over 100 thymectomies in rats about the age of puberty or younger. In the course of this work, it was frequently noted that when a small, non-traumatized fragment of the gland was accidently left behind at operation, autopsy disclosed a fairly large regenerated mass, even as early as 2 weeks after the partial thymectomy. But this evidence was indirect as we had no means of accurately measuring the fragment accidently left. These incidental findings suggested that the gland was probably capable of undergoing very rapid compensatory hypertrophy as well as regeneration.

A review of the extensive literature on the thymus shows that no positive evidence of uncomplicated compensatory hypertrophy has been obtained.

Fulci (4) while primarily interested in thymus cytology, emphasized the regenerative capacity of the thymus and pointed out that the gland was capable of undergoing enlargement after partial removal. He operated on a series of rabbits, removing large portions of the thymus gland, and studied the cytological changes which occurred in the remaining fragments from a few hours to several

* Read before the American Association of Pathologists and Bacteriologists, Washington, D. C., May 5, 1925.

months after operation. In many of his operations he used a cautery to control bleeding. His regenerative studies were, therefore, complicated because the remaining fragment was traumatized.

The method of transplantation of the thymus has been used by Tongu (5), Grimani (6), Marine and Manley (7), Scalone (8), Flesch (9), and Yamanoi (10) in the study of regeneration. We ourselves have studied a large series of autothymus transplants from 1 to 70 days old in the rat. While all these studies indicate that the thymus transplants take and grow, they have very little bearing on the question of compensatory hypertrophy. From the clinical side there is abundant evidence that the thymus is capable of undergoing regeneration and probably also compensatory hypertrophy. The enlarged thymus that one sees in Addison's disease, Graves' disease, acromegaly, and status thymicolymphaticus lends support to this view.

Methods.

All rats were reared in the laboratory from very tame albino stock (*Mus norvegicus albinus*), obtained from the Wistar Institute. Wistar rats offer the advantage that growth and developmental curves for the body as a whole and for the various organs have been tabulated.

The young are weaned at about 25 days of age and are then given a completely adequate diet, consisting of 15 parts rolled oats, 60 parts hominy, 14 parts dried meat scrap, 10 parts dried milk powder, and 1 part salt. This diet is supplemented with bread and fresh milk daily until the animals are 45 days of age, after which bread and milk are given once a week, fresh vegetables are fed two and three times a week, and all rats are given fresh tap water daily. The animals are fairly free of snuffles and chronic pneumonia.

Our operative technique was standardized. The operative field was prepared by shaving and this was followed by wiping the area with sponges dipped in bichloride solution and alcohol. The sternum was cut by a midline incision, the thymus area exposed, and the right lobe was gently and quickly separated from the left lobe and excised. Ether anesthesia was always used, by the open method. Food was withheld about 12 hours before operating and the rats were not fed for 12 hours after operation.

At the end of the 2nd day the operated rats were placed in large cages together with their controls. The experimental and control rats were kept under identical laboratory and nutritional conditions at all times, this being of the utmost importance in any study of the thymus gland. For the first 24 hours after the operation the operated rats received some special care.

EXPERIMENTAL DATA.

Three groups of experiments, two on young rats before the age of puberty and one on a small number of animals after puberty, were

carried out in order to obtain direct proof of compensatory hypertrophy. The animals in each group were divided equally. In one-half of each group the right lobes were removed, while the remaining animals were used as controls. A litter control of the same sex was kept for each operated animal. It may be mentioned here that the rat is well adapted for this work. Both lobes of the main thymus (arising from the third gill arches) are practically of the same size, mainly intrathoracic, and they can be easily separated and removed at operation.

TABLE I.

No.	Sex.	Age when killed.	Body weight when killed.	Length of time after removal of right lobe.	Weight of left lobe.	Weight of right lobe.	Résumé.
		days	gm.	*days*	*mg.*	*mg.*	
RW92-2	F.	65	100	15	189	Removed.	Operated rats: Average weight, left lobes..178 mg.
RW92-3	"	65	102	15	176	"	
RW92-4	M.	65	122	15	205	"	Control rats: Average weight, left lobes..130 mg.
RW93-1	F.	65	101	15	197	"	
RW93-2	"	65	98	15	124	"	Percentile difference, in favor of operated rats .....37 per cent
RW93-3	"	65	93	Control.	134	118	Average difference in weight
RW93-4	M.	65	120	"	159	151	between right and left
RW94-1	F.	65	105	"	110	122	lobes of controls..3 per cent
RW94-2	"	65	104	"	113	116	Difference in body weight,
RW94-3	"	65	107	"	134	125	operated and control rats .....1 per cent

The animals were killed 15 to 24 days after the beginning of these experiments, and the thymus glands were dissected from the surrounding fat and lymph nodes. The individual lobes were weighed immediately.

Group I (*See Table I*).

The first group of animals, consisting of five operated rats and five controls under the age of puberty, were autopsied 15 days following removal of the right lobe. The left lobes of the operated animals averaged 37 per cent in weight above the left lobes of the controls. The average difference in weight between the right and left lobes of

the controls was only 3 per cent. The difference in body weights between the controls and the experimental rats was only 1 per cent. It is noteworthy that this marked hypertrophy of the left lobe, following removal of the right lobe, occurred in 15 days.

Group II (See Table II).

In the second group of experiments, right lobectomies were carried out on rats before puberty. These rats were a little younger at operation than the animals in Group I. They and their controls were killed

TABLE II.

No.	Sex.	Age when killed.	Body weight when killed.	Length of time after removal of right lobe.	Weight of left lobe.	Weight of right lobe.	Résumé.
		days	*gm.*	*days*	*mg.*	*mg.*	
RW88-2	F.	69	135	24	184	Removed.	Operated rats: Average weight of left lobes. 197 mg.
RW88-3	M.	69	160	24	234	"	
RW88-4	F.	66	120	21	185	"	Control rats: Average weight of left lobes.....138 mg.
RW89-1	"	69	115	24	186	"	Percentile difference in favor of the operated rats..... .....42 per cent.
RW89-2	"	66	125	Control.	130	126	Average difference in weight between right and left lobes of controls.....5 per cent.
RW89-3	"	69	135	"	134	140	
RW89-4	M.	69	150	"	169	153	
RW90-1	F.	69	135	"	119	135	Difference in body weights between operated and control rats, in favor of controls.....3 per cent.

from 3 to 4 weeks after the beginning of the experiment. The left lobes of the operated animals were 42 per cent heavier than the left lobes of their controls. In this group also there was a difference of only 5 per cent between the right and left lobes of the control animals, and 3 per cent in the body weights. It will be noted that the hypertrophy in this group is apparently greater than in Group I. If this is confirmed it may be attributed to the fact that the animals were somewhat younger than those of Group I, and in addition there was a greater time interval between operation and examination of the

thymuses. However, with such a small series the difference may be only accidental.

Group III.

Next, we took up the question of compensatory hypertrophy of the thymus after puberty. Some experiments carried out about a year ago on old rats, which were partially thymectomized and killed 2 weeks after operation, showed that regeneration did not take place. This conclusion was based upon the comparison of the microscopic appearance of sections made from the tissue removed at the time of the partial thymectomy, with those made from the gland which was removed 2 weeks later (2).

In our present work we found that removal of one lobe in rats past puberty does not induce a compensatory enlargement of the other lobe. We arrived at our results in two ways. First, we removed one lobe, weighed it immediately, and compared its weight with the remaining lobe when the animal was killed 2 weeks later. We also carried out unilateral lobectomies and compared the remaining lobes of the operated animals with the corresponding lobes of the controls.

DISCUSSION AND CONCLUSION.

Previous experimental work concerning enlargement of the thymus has dealt mainly with the regeneration which follows partial or subtotal removal, or with the regeneration which follows transplantation. We planned our experiments to obtain evidence of uncomplicated hypertrophy by removing one lobe and studying the enlargement of the remaining lobe. Our results clearly indicate that such hypertrophy takes place in young animals. They emphasize the functional importance of this gland before puberty, since the compensatory enlargement does not take place after puberty. The results further indicate that with the onset of involution there is a marked or almost total decline in the function of the gland, in spite of the fact that anatomically the thymus may persist to old age. With the appearance of a stimulus for regeneration, as, for instance, suprarenalectomy, the involuted gland takes on functional activity.

We wish to express our appreciation of the stimulating criticism of Dr. David Marine.

BIBLIOGRAPHY.

1. Jaffe, H. L., The influence of the suprarenal gland on the thymus. I. Regeneration of the thymus following double suprarenalectomy in the rat, *J. Exp. Med.*, 1924, xl, 325.
2. Jaffe, H. L., The influence of the suprarenal gland on the thymus. II. Direct evidence of regeneration of the involuted thymus following double suprarenalectomy in the rat, *J. Exp. Med.*, 1924, xl, 619.
3. Jaffe, H. L., The influence of the suprarenal gland on the thymus. III. Stimulation of the growth of the thymus gland following double suprarenalectomy in young rats, *J. Exp. Med.*, 1924, xl, 753.
4. Fulci, F., Die Natur der Thymusdrüse nach Untersuchungen über ihre Regenerationsfähigkeit bei den Säugetieren, *Deutsch. med. Woch.*, 1913, xxxix, 1776.
5. Tongu, Y., Recherches expérimentales sur la transplantation du thymus. (Première étude.), *Mitt. med. Fakult. k. Univ. Tokyo*, 1915, xiv, 259.
6. Grimani, E., Sulgi effetti del trapianto del timo e sulle correlazioni funzionali fra testicolo e timo, *Arch. anat. pat.*, 1905, i, 351.
7. Marine, D., and Manley, O. T., Transplantation of the thymus in rabbits—relation of the thymus to sexual maturity, *J. Lab. and Clin. Med.*, 1917–18, iii, 48.
8. Scalone, I., Ulteriori ricerche sul trapianto del timo. (Trapianto negli organi interni), *Gazz. internaz. med.*, 1910, xiii, 86, 101.
9. Flesch, M., Jr., Experimentelle Thymusstudien. 1. Teil. Thymus und Milz bei der Ratte, *Beitr. klin. Chir.*, 1915, xcv, 376.
10. Yamanoi, S., Über autoplastische Transplantation der Thymus in die Milz bei Kaninchen, *Frankf. Z. Path.*, 1921–22, xxvi, 356.

STUDIES ON THE BACTERIOPHAGE OF D'HÉRELLE.

II. Effect of Alcohol on the Bacteriophage of d'Hérelle.

By JACQUES J. BRONFENBRENNER, Ph.D., and CHARLES KORB, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 6, 1925.)

In the course of some experiments in which we had occasion to precipitate lytic filtrates with alcohol, we observed that in many instances such precipitates manifested appreciable lytic action. Because of the divergent statements in the literature upon this point and the interest which the effect of alcohol on the lytic principle might have in connection with the question of the animate nature of the bacteriophage, we have attempted a brief inquiry into this question.

D'Hérelle[1] has reported that by precipitating a filtrate of antidysentery bacteriophage with 9 volumes of 96 per cent alcohol, decanting the supernatant fluid after 48 hours, and redissolving the precipitate in saline, he obtained a substance slightly lytic for dysentery bacilli. This lytic action, however, was not transmissible in series. D'Hérelle concluded that the alcohol destroyed the living ultramicrobe (bacteriophage) and precipitated its endoenzyme which was responsible for lysis in the first generation. Hauduroy,[2] repeating the experiment of d'Hérelle, obtained a similar result by precipitating sterile bouillon with alcohol. He suggested, therefore, that the apparent lytic action of the precipitate obtained by d'Hérelle was due to the bacteriostatic effect of the alcohol adsorbed by this precipitate. Complete destruction of the lytic activity of bacteriophage by alcohol was also observed by Watanabe[3] and by Arnold.[4]

On the other hand, in the course of some other work, Kabéshima[5] states that he has separated bacteriophage from its solution by precipitation with alcohol. This author, however, does not indicate the concentration of alcohol used nor the

[1] d'Hérelle, F., Le bactériophage. Son rôle dans l'immunité, Monographies de l' Institut Pasteur, Paris, 1921, 95.

[2] Hauduroy, P., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 964.

[3] Watanabe, T., *Arch. Hyg.*, 1923–24, xcii, 1.

[4] Arnold, L., *J. Lab. and Clin. Med.*, 1922–23, viii, 720.

[5] Kabéshima, T., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 219.

length of exposure. Appelmans,[6] too, found that bacteriophage action is not completely destroyed even after 20 days exposure to alcohol in 50 per cent concentration. He does not state whether the lytic action observed by him is transmissible in series, or, like that observed by d'Hérelle, is demonstrable in first generation only.

Our experiments were first made with two strains of bacteriophage active against *Bacillus dysenteriæ* Shiga and Flexner respectively.[7] Later, similar results were obtained with a bacteriophage active against colon bacillus and with two strains of bacteriophage active against *Bacillus enteritidis*, M. T. I; and *Bacillus pestis caviæ* M. T. II respectively.[8]

The general procedure adopted in the experiments was as follows:

Several portions of 1 cc. each of active filtrate were carefully deposited at the bottom of sterile centrifuge tubes by means of a pipette in such a way as to exclude any contact with the walls of the upper portion of the tubes. Into each tube was then introduced 9 cc. of 95 per cent alcohol, and after thorough mixing of the contents, the mixtures were overlaid carefully with 1 cc. more of 95 per cent alcohol to exclude the possibility of deposition of droplets of the lytic principle on the walls of the tube above the surface of the alcohol. The tubes were kept at room temperature and at stated intervals the contents were centrifuged for 10 minutes, the liquid decanted, and the precipitate taken up in 1 cc. of sterile physiological salt solution. Both the alcohol fraction and the redissolved precipitate were titrated for their respective lytic power by the tenfold dilution method of Appelmans[6] as modified by Werthemann.[9] All dilutions for titration were made in broth adjusted to a slightly alkaline reaction (pH = 7.4) and containing about 1,000,000 young susceptible bacteria in each cc. A fresh sterile pipette was used for each dilution. The presence of active principle in the tubes exhibiting a doubtful degree of lysis or a possible overgrowth of resistant bacteria was at first controlled in the usual manner—by transfer to agar slants previously seeded with susceptible bacteria. Later it was found more satisfactory to incubate such tubes for 36 to 48 hours, thus allowing the lytic titer to reach its maximum in all the tubes where there was any bacteriophage present. At the end of this time the

[6] Appelmans, R., *Compt. rend. Soc. biol.*, 1921, lxxxv, 1098.

[7] These strains of bacteriophage were isolated by us from stools obtained from the Babies' Hospital through the kind cooperation of Dr. Martha Wollstein.

[8] The last two strains of bacteriophage were selected on account of their high specificity among seven strains isolated by us from stools of mice surviving the experimental infection and kindly placed at our disposal by Dr. Leslie T. Webster and Dr. Ida W. Pritchett.

[9] Werthemann, A., *Arch. Hyg.*, 1922, xci, 255.

Protocol 1.

The Effect of Alcohol on the Lytic Activity of Bacteriophage at Room Temperature

Laudman Shiga bacteriophage	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.
95 per cent alcohol	0.0 " (control).	10.0 "	10.0 "	10.0 "	10.0 "	10.0 "

Kept at room temperature for periods indicated below; centrifuged for 10 min. after time as stated; precipitate taken up in 1 cc. 0.85 per cent salt solution; alcohol-soluble fraction (supernatant fluid) and the precipitated fraction titrated separately.

Length of exposure to alcohol				10 min.				1¼ hrs.				3 hrs.				6 hrs.				24 hrs.			
				Supernatant.			Precipitate.	Supernatant.			Precipitate.	Supernatant.			Precipitate.	Supernatant.			Precipitate.	Supernatant.			Precipitate.
	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Serial lysis.
cc.																										
10^{-1}	+						+	+	+			+	+	+												
10^{-2}	+			+	+	+	+	+	+	−	−	+	+	+	−	−	−	+	+	+	−	−	−	−	−	−
10^{-3}	+			+	+	+	+	+	+	−	−	+	+	+	−	−	−	+	+	+	−	−	−	−	−	−
10^{-4}	+			−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10^{-5}	+			−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10^{-6}	+			−	−	−	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10^{-7}	+			−	−	−	−	−	−	−																
10^{-8}	+	+	+	−			−	−																		
10^{-9}	+	+	+																							
10^{-10}	+	+	+																							
10^{-11}	−	−	−																							

+ = lysis of bacteria; − = no lysis.

tubes were heated for 30 minutes at 56°C. and their contents were tested for lytic activity by transferring 0.1 cc. from each of these tubes into broth seeded with susceptible bacteria. In all cases where bacteriophage was present the lysis took place in 3 to 4 hours and was transmissible in series.

The protocol of one of these experiments (Protocol 1) is given above. With slight modifications, which will be indicated, all the experiments followed the general scheme outlined in this protocol. The repetition of the protocol will therefore be omitted, and the results will be tabulated in terms of minimum amount of solution exhibiting lytic activity. The results shown in Protocol 1 are tabulated in Table I.

TABLE I.

Inactivation of Bacteriophage by Alcohol at Room Temperature.

1 cc. Laudman Shiga bacteriophage + 10 cc. 95 per cent alcohol at room temperature.

Exposure to alcohol for.....	10 min.	1¼ hrs.	3 hrs.	6 hrs.	24 hrs.	
Lytic titer of supernatant alcohol.....	10^{-3} cc.	Not active in 10^{-2} cc.	Not active in 10^{-2} cc.	Not active in 10^{-2} cc.	Not active in* 10^{-2} cc.	
Lytic titer of precipitate....	10^{-5} cc.	10^{-3} cc.	10^{-3} cc.	10^{-3} cc.	Not active in 10^{-1} cc.	
Original lytic titer.....						10^{-10} cc.

* The supernatant alcohol was not tested in amounts above 0.1 cc. corresponding to 0.01 cc. (or 10^{-2} cc.) of the original filtrate to avoid bacteriostatic effect of alcohol.

From Table I it is seen that only a trace of lytic principle was demonstrable in the supernatant fluid after the first 10 minutes of contact with alcohol, while in the precipitate measurable activity persisted for over 6 hours. Moreover, as long as this lytic activity persisted, it was consistently transmissible in series. We were unable to observe the non-transmissible lysis ascribed by d'Hérelle[1] to the action of an endoenzyme. Thus it appears that the bacteriophages we employed are completely inactivated by contact with 10 volumes of 95 per cent alcohol at room temperature in less than 24 hours.

D'Hérelle lays great stress on this susceptibility of bacteriophage to the action of alcohol as one of the important links in the chain of evidence in favor of its animate nature. While it is true that if the bacteriophage were a living parasite one would expect it to be affected by alcohol, as is the case with most if not all living organisms,[10] yet the converse does not necessarily follow. The inactivation of bacteriophage by alcohol does not necessarily indicate its living nature since alcohol may conceivably denature an inanimate organic substance sufficiently to modify or destroy some of its properties. Certain enzymes, for instance, are very quickly inactivated by the alcohol.[11] Alcohol also destroys the potency of bacterial toxins in general, and of *botulinus* toxin in particular.[12] While this inactivation is most rapid at higher temperatures, we observed that even at 5°C. the potency of *botulinus* toxin is reduced 30,000 times in 10 minutes.[13]

Suspecting that the action of alcohol on bacteriophage may have the character of such a denaturation, we thought that perhaps at a lower temperature this denaturation would be sufficiently delayed to permit a close study of the process.

With this in view, the above experiment was repeated in every detail except that both the lytic filtrate and the alcohol were cooled, prior to mixing, and were left, after mixing, at the same temperature (6–7°C.) for different periods of time, as indicated in Table II.

At stated intervals the mixtures were centrifuged, the supernatant fluid was separated from the precipitate, and both were titrated by serial dilution as before. It will be seen from Chart 1 by comparing the results recorded in Table II with those of the preceding experiment (Table I) that, whereas at room temperature complete inactivation of lytic power of the filtrates by alcohol required only about 1 hour for the supernatant fluid and more than 6 hours for the precipitable fraction, at ice box temperature the supernatant fluid exhibited

[10] Rous sarcoma virus according to Funk, as well as virus of mosaic disease of plants according to Iwanowski (Iwanowski, D., *Centr. Bakt., 2. Abt.*, 1899, v, 250), is not affected by alcohol.

[11] Hudson, C. S., and Paine, H. S., *J. Am. Chem. Soc.*, 1910, xxxii, 1350.

[12] Bronfenbrenner, J., and Schlesinger, M. J., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 304.

[13] Bronfenbrenner, J., unpublished experiments.

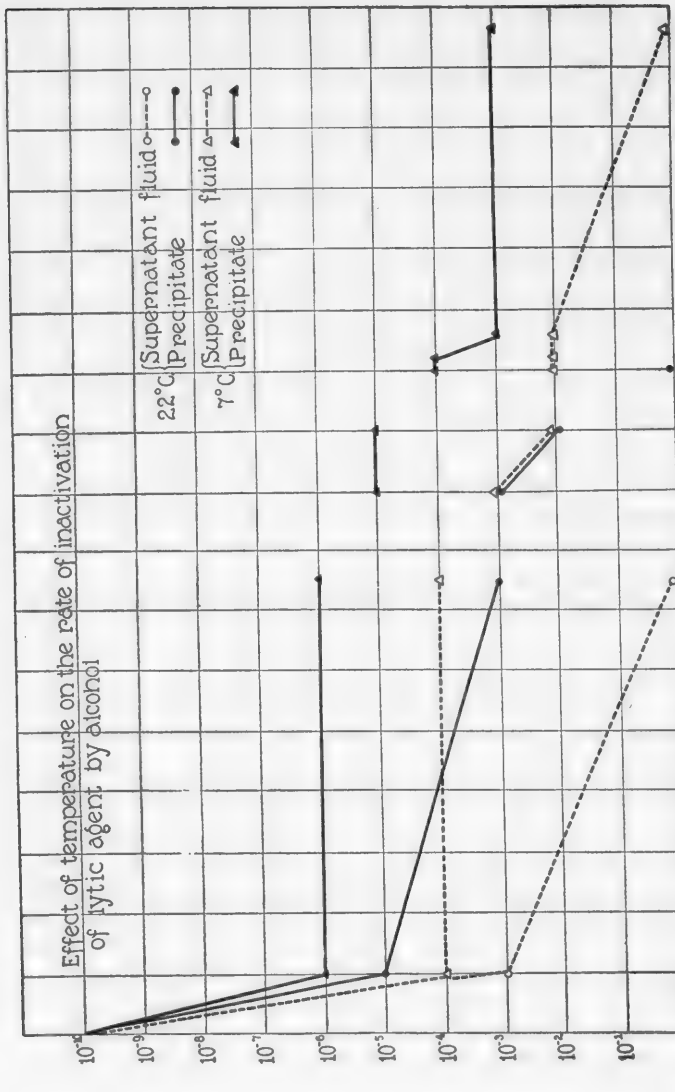

Effect of temperature on the rate of inactivation of lytic agent by alcohol
22°C Supernatant fluid
Precipitate
7°C Supernatant fluid
Precipitate
Min. 0 10 20 30 40 50 60 70 Hrs. 3 6 Days 5 10 15 20 25

CHART 1.

some degree of activity even after 4 days, and the precipitate still caused serial lysis after the exposure to ten times its volume of 95 per cent alcohol for 4 weeks.

These experiments were repeated several times and with a number of different lytic filtrates with similar results, notably at ice box temperature. At room temperature the time for complete loss of lytic activity of the precipitate varied with different filtrates from 3 to 24 hours.

While the final inactivation of the lytic filtrates by the alcohol was slower at ice box temperature, it is to be noted that almost the same marked initial reduction of lytic activity took place at ice box as at

TABLE II.

Inactivation of Bacteriophage by Alcohol at 7°C.

1 cc. Laudman Shiga bacteriophage + 10 cc. 95 per cent alcohol at ice box temperature (+7°C.).

Exposure to alcohol for.....	10 min.	1¼ hrs.	3 hrs.	6 hrs.	24 hrs.	44 hrs.	4 days.	28 days.	
Lytic titer of supernatant alcohol.....	10^{-4} cc.	10^{-4} cc.	10^{-3} cc.	10^{-2} cc.	10^{-2} cc.	10^{-2} cc.	10^{-2} cc.	Not active in 1×10^{-2} cc.	
Lytic titer of precipitate.....	10^{-6} cc.	10^{-6} cc.	10^{-5} cc.	10^{-5} cc.	10^{-4} cc.	10^{-4} cc.	10^{-3} cc.	10^{-3} cc.	
Original lytic titer....									10^{-10} cc.

room temperature.[14] Since the tubes, in order to bring about titration of their contents, had to be taken into the room to be balanced preliminary to centrifuging, and then centrifuged, it was thought that in these 15 to 20 minutes the change in the temperature of the mixtures might be sufficient to account for the rapid initial inactivation almost equal to that occurring when the entire process was carried out at room temperature.

In order to prevent this rise in temperature of the cooled bacteriophage-alcohol mixtures during centrifuging, this latter step was

[14] Bronfenbrenner, J., and Korb, C., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 177.

omitted. After varying intervals the precipitate which appeared following the addition of alcohol to the lytic filtrate was thoroughly suspended by repeated aspiration into a pipette. From the resulting homogeneous suspension, 0.1 cc. was transferred each time directly into broth for titration by further dilution in series, as in the previous tests.

The results of this titration indicate that when the temperature was kept at 7°C. throughout the experiment, the inactivation of the filtrate by the alcohol was appreciably slower than at room temperature. However, even under these circumstances, lytic activity of the filtrate was reduced to 1 per cent of its original value within 1 hour after the exposure to alcohol (Table III).

TABLE III.

Combined Lytic Activity of the Supernatant Fluid and of Sediment after Exposure to Alcohol at 7°C.

1 cc. Laudman Shiga bacteriophage + 10 cc. 95 per cent alcohol at 7°C.

Exposure to alcohol for..	15 min.	1 hr.	3 hrs.	4½ hrs.	7 hrs.	24 hrs.	48 hrs.	4 days.	12 days.	24 days.	
Lytic titer of the mixture.....	10^{-9} cc.	10^{-8} cc.	10^{-7} cc.	10^{-7} cc.	10^{-6} cc.	10^{-5} cc.	10^{-4} cc.	10^{-3} cc.	10^{-3} cc.	10^{-3} cc.	
Original titer of the phage.....											10^{-10} cc.

Rate of Destruction of Bacteriophage on Second Precipitation by Alcohol.

While the loss of lytic activity in the preceding experiment was equal to 99 per cent in the 1st hour after exposure to alcohol (from lytic titer of 10^{-10} cc. to 10^{-8} cc.), further reduction of activity seemed to take place at a progressively slower rate.

Since it has been suggested in the literature[15,16] that lytic filtrates may consist of several component fractions differing in their respective

[15] Kouo-Ngen, J. T., and Wagemans, J., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 1253; 1923, lxxxviii, 303.

[16] Reichert, F., *Centr. Bakt., 1. Abt., Orig.*, 1923–24, xci, 235.

resistance to injurious agents, the possibility that the greater initial rate of destruction of bacteriophage by the alcohol may be due to the presence of a certain fraction which is particularly susceptible to the action of alcohol was inquired into.

Two portions of cooled lytic filtrate, of 1 cc. each, were placed in two centrifuge tubes, precipitated with 10 cc. of cooled alcohol, and placed at 7°C. After stated periods the contents of each tube was thoroughly mixed and 0.1 cc. samples were taken out for titration to determine the rate of inactivation of the lysin. After 20

Protocol 2.

Inactivation of Bacteriophage by Repeated Treatment with Alcohol at 7°C.

	cc.	cc.	cc.
Cooled filtrate	1	1	1
Cooled 95 per cent alcohol	10	10	—
Physiological salt solution	—	—	10
Placed at 7°C. and titrated at intervals.			
Initial lytic titer			10^{-10}
Lytic titer after 2 hrs.	10^{-8}	10^{-8}	
" " " 4 "	10^{-7}	10^{-7}	
" " " 20 "	10^{-6}	10^{-6}	
Centrifuged at 10°C. Precipitate taken up in 1 cc. of cooled salt solution.			
Lytic titer (20 hrs.)	10^{-6}		
Cooled 95 per cent alcohol	10		
Placed at 7°C.			
Lytic titer after 24 hrs.	10^{-3}	10^{-5}	
" " " 42 "	10^{-2}	10^{-4}	10^{-10}

hours of contact with alcohol, one of the tubes was subjected to centrifuging (in a specially cooled centrifuge). The precipitate thus obtained was separated from the supernatant alcohol and taken up in 1 cc. of cooled physiological salt solution. A sample (0.1 cc.) of this solution was transferred into broth for immediate titration to determine the extent of its activity at that time, and the remaining solution was again precipitated by 10 volumes of 95 per cent alcohol, and replaced in the ice box. At intervals the contents of this tube, as well as those of the second tube (which remained undisturbed in the ice box, as control), were thoroughly mixed and samples taken for titration by serial dilution.

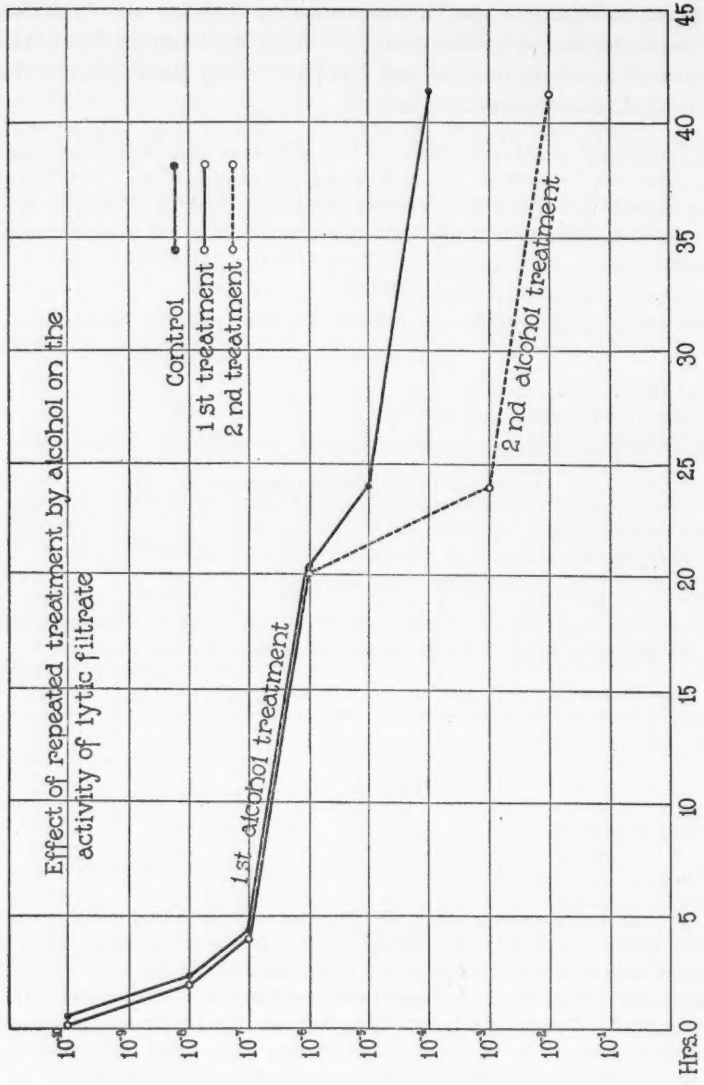

Effect of repeated treatment by alcohol on the activity of lytic filtrate
Control
1 st treatment
2 nd treatment
1st alcohol treatment
2 nd alcohol treatment
Hrs. 0
5
10
15
20
25
30
35
40
45
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}

CHART 2.

The results of these titrations are plotted in Chart 2 from the data presented in Protocol 2. As can be seen the inactivation of lysin in the first period of the experiment went on in the same way in both tubes. During the first 2 hours of contact with alcohol there was a one hundredfold diminution in the activity of the lysin which further decreased tenfold in the 2 hours following. When, 20 hours later, the remaining lysin was again subjected to the action of alcohol, the rate of its deterioration duplicated that observed in the initial stages of the first precipitation with alcohol.

It appears, therefore, that the susceptibility of the lytic principle to alcohol is uniform throughout, and that the observed difference in the rate of deterioration at the later stages of exposure is not due to the presence of a more resistant fraction.

SUMMARY AND CONCLUSIONS.

When bacteriophage is precipitated by alcohol at room temperature its activity rapidly and progressively decreases until it is totally destroyed, between 6 and 24 hours after exposure.

If the percipitation is carried out at 7°C. the destruction of lytic activity is considerably slower; measurable traces may be detected even after 4 weeks exposure to alcohol. Although the major portion of the lytic activity is found in the precipitate, the supernatant alcohol carries a measurable amount of lytic principle which remains active for several days.

In all cases the residual lytic activity was found to be transmissible in series. In no instance were we able to observe the non-transmissible action ascribed by d'Hérelle to the enzyme.

The persistence of traces of active principle after many weeks of exposure to alcohol at low temperature is not found to be due to the existence in the original filtrate of a fraction relatively resistant to the effect of alcohol.

The inactivation of bacteriophage by alcohol seems, therefore, analogous to the alcoholic inactivation of certain enzymes and toxins.